CHARACTERISATION OF *COWDRIA RUMINANTIUM* (AGENT OF HEARTWATER INFECTION) ISOLATES FROM KENYA.

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DECLARATION

The work presented in this thesis is all my own except where it is indicated and collaboration is gladly acknowledged.

DEDICATION

Kûrí Baba munjiari Mr. William Ngùmi Njoroge ûría wendire ndachûke maitho, na Maitû mûnjiari Mary Njeri Ngûmi, ni wendo wake mûnene kûbaríríra maûndûiní mothe, na mwana wakwa Paul Ngûmi Kíama we ûtûmíte ngíe na kíyo gía gwíka maûndû maya.

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LIST OF ABBREVIATIONS

µl	microlitre
µM	micromolar
ab	antibody
AEZ	agro-ecological zone
ag	antigen
APC	antigen presenting cell
APS	ammonium persulfate
BLP	Buffered Lactose Peptone
C	degrees Celsius
C3b	third factor of complement
cELISA	competitive enzyme linked immuno sorbent assay
CR	complement receptor
CTL	cytotoxic lymphocyte
CTVM	Centre For Tropical Veterinary Medicine
dH2O	distilled water
DI	diagnostic index
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
e.g.	for example
EBs	elementary bodies
ECF	East Coast fever
EDTA	ethylene diamine tetracetic acid
EtBr	Ethidium bromide
FAO	Food and Agriculture Organisation
FAT	Fluorescent antibody test
GIT	gastrointestinal tract
GMEM	Glasgow's Minimum Essential Medium
HCl	hydrochloric acid
HRP	horse radish peroxidase
i.e.	that is
i/m	intramuscular
i/v	intravenous
iELISA	indirect enzyme linked immuno sorbent assay
IFAT	Indirect fluorescent antibody test
IFN-γ	interferon gamma
IgG	immunoglubulin gamma
IL	inhibition level

IL-2	interleukin two
ILRAD	International Laboratories for Research in Animal Diseases
ILRI	International Livestock Research Institute
kDa	Kilodalton
KARI	Kenya Agricultural Research Institute
MEM	Minimum Essential Medium
mg	milligrams
MHC	major histocompatibility complex
ml	millilitre
mM	millimolar
MOH	mouse organ homogenates
MQ H2O	MilliQ water
no.	number
NVRC	National Veterinary Research Centre (Muguga)
OD	optical density
OTC	oxytetracycline
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing Tween 20 specified as a percentage
PCR	Polymerase chain reaction
pi	post inoculation
RI	reaction index
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
s/c	subcutaneous
SDS	sodium dodecyle sulphate
SDS-PAGE	sodium dodecyl sulphate - poly acrylamide gel electrophoresis
SR-CRSP	Small Ruminant - Collaborative Research Support Program
TBE	Tris/boric acid/EDTA buffer
TC	tissue culture
TEMED	N,N,N′,N′-tetramethylethylene diamine
TLA	long acting terramycin
TMB	Tetramethyl benzidine
Tween 20	polyoxyethylene sorbitan monolaurate
UK	United Kingdom
w/v	weight per volume

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SUMMARY

Cowdria ruminantium (Rickettsiales) causes heartwater, a disease of high mortality in susceptible ruminants. A description of the isolation of new *Cowdria* isolates by different methods and from different vectors and geographical locations in Kenya is given. These included *Amblyomma variegatum, A. gemma and A. lepidum.* Isolates from the later two species were also by feeding adults moulted from nymphs collected in the field and is the first report on transtadial transmission by *A. gemma* ticks. A spectrum of virulence ranging from highly virulent to mildly virulent for sheep was found among the *Cowdria* isolates. The majority of isolates were highly virulent. There was a range of mouse infectivity among the isolates from inapparent to lethal. The Asembo and Baragoi isolates were pathogenic and lethal, the Kiswani, was infective and non pathogenic for Balb/C mice while the other 8 were avirulent or refractile to mice inducing only antibody production in various proportions of mice. There was a difference in the infectivity for neutrophils both in the frequency of infected cultures and in their level of infection. The different isolates were classified as of low infectivity where even the few positive cultures rarely reached 1% infection rate, medium infectivity if a good number of cultures regularly attained 1% infected neutrophils, or high infectivity if a large proportion of the cultures became positive regularly attained 1% infected neutrophils and at least some of them attained more than 10% infected neutrophils. The isolates also had a range of infectivity for the brain endothelial cells, from no detectable colonies to greater than 16% infected endothelial cells in individual animals. A diagnostic index, that is the ease by which diagnosis could be made at post mortem (% endothelial cells where colonies were found), for each isolate by brain crush smears was formulated and virulent isolates generally had a high diagnostic index whereas low virulent isolates had lower diagnostic indices, the correlation between RI and DI being highly significant ($p < 0.001$ Spearmans correlation coefficient $r_s = 0.957$). All isolates were confirmed to be *Cowdria* by PCR and western blots. A one way cross immunity was performed with all isolates using animals that had been treated or that had naturally recovered during the infection studies. A spectrum of protection was seen ranging from zero to complete protection.

A two way cross immunity was performed with five isolates which had been selected on the basis of their differences in virulence, (two milder and three virulent for sheep), tick source and diverse geographical origin, four isolates were found to confer different degrees of overall protection against the other isolates. In the order of their ascending virulence, Bamba, Asembo, Baragoi and Suswa, gave, 18.8%, 56.1%, 98.8% and 67.0% cross protection respectively. Therefore the least virulent gave the least overall protection while the second most virulent (the Baragoi isolate) gave the broadest overall protection against all the other isolates. This supports the suggestion that immunogenicity is closely related to the pathogenicity and virulence of *Cowdria*. It also implies that total immunogenicity may be found in one rather than a cocktail of stocks. Neither of the low virulence nor the high virulence isolates may be an acceptable vaccine. However, the one way trial indicated that one of the intermediate virulence isolates the Marigat isolate should be evaluated as it was protective against the Baragoi isolate with a 98.5% reduction in the reaction index and be safer for use in sheep. The two main vector ticks in Kenya, *A variegatum* and *A gemma* were examined for their susceptibility to infection with *Cowdria* (Asembo and Bamba) isolated from both species respectively. However infection rates were very low for both isolates to both species and no conclusions could be drawn, perhaps because the *Cowdria* isolates selected were both the milder ones.

The author concludes that the agent of heartwater is endemically widespread in many districts in Kenya and poses a potential threat of outbreaks to areas newly invaded by vector ticks and also to areas where immunity due to the local agent may not protect against an invading one. The author recommends that the Baragoi or Marigat isolate should be adopted for possible vaccine development in Kenya.

GENERAL INTRODUCTION AND DESIGN OF EXPERIMENT

Heartwater is a non-contageous, multihost disease that is particularly virulent, to cattle, sheep and goats, but is milder or inapparent to other hosts. It is characterised by a high fever and nervous symptoms and sudden death. Hydropericardium is a common post mortem lesion from which the desease was named but it is not always present. The causal agent, *Cowdria ruminantium* (Cowdry, 1925a), is a rickettsia, whose multiple vectors belong to ixodid ticks of the genus *Amblyomma* (Cowdry, 1925b). It occurs in most of sub-Saharan Africa, islands off shore the African continent, Madagascar and some Caribbean islands (Camus and Barre, 1982; Provost and Bezuidenhout, 1987).

Heartwater was first diagnosed in South Africa by Lois Trichardt in, 1838, in his sheep three weeks after they had suffered a heavy tick infestation (quoted by Neitz, 1947, cited by Henning (1956). However the source of infection remained unknown until Lounsbury (1900), demonstrated that the disease could be transmitted by the South African bont tick, *Amblyomma hebraeum*. The causative agent remained undiscovered for another two and a half decades when Cowdry (1925a & b and 1926a & b) elucidated it in the tissues of the host and of the tick. Cowdry named the etiologic agent *Rickettsia ruminantium* but it was renamed *Cowdria ruminantium*, by Moshkovski (1947) who had proposed it as a subgenus due to inability to grow it in laboratory animals and in tissue culture (Moshkovski, 1945).

In Southern Africa it is the most important tick-borne disease of livestock (Howell *et al.* (1981), while in Eastern Africa it ranks second to East Coast fever among four main tick borne diseases that constrain livestock production and development in the region (Uilenberg, 1981). Heartwater, like other tick borne diseases is particularly virulent to exotic animals and their crosses. Wild ruminants are also affected but are thought to act primarily as reservoirs of infection (Henning, 1956; Oberem and Bezuidenhout, 1987a).

A number of curative drugs have been discovered and therapeutics have been readily available for a long time, but the acuteness of the disease usually pre-empts their use.

Cultivation of the organism *in vitro* was not possible until recently (Bezuidenhout *et al.*, 1985), and could not be adapteted to grow routinely in laboratory animals until,

Du Plessis and Kumm, (1970), discovered a mouse pathogenic isolate. The importance of cowdriosis has been unrealised as the disease is inapparent in the indigenous herds in many African countries. There is difficulty of confirming infection in the live animal and specific and reliable serological tests (Du Plessis and Malan, 1987a), have not been available.

As a result, with the exception of South Africa and Zimbabwe, the disease has remained poorly understood in many African countries. But there has been renewed interest with the discovery that it exists in the Caribbean islands and the realisation of its potential threat to the American mainland. However, general interest has been awaked in more African countries because of technological advancements, and research programmes on heartwater are in West Africa, East Africa as well as laboratories in Europe and America. The economic cost of the disease has at best been given conservative estimates (Camus *et al., 1*996), in most countries where it occurs. The disease, together with other tick borne diseases, have been the main impediments to the introduction of high yielding dairy cattle to many high potential zones in African countries.

A blood vaccine produced in South Africa, is used extensively in South Africa, Zimbabwe and Malawi but lack of protection in some immunised animals under field challenge has been reported even within South Africa (Du Plessis *et al., 1*989).

Heartwater in Kenya was first reported by Daubney, (1930), but data on the distribution of the disease is still sparse and not well documented (Njenga and Mugera, 1989). Many reports of sporadic heartwater cases appear in the Director of Veterinary Services Annual Reports in Kenya (Anon, 1981a), whereas there is one solid account concerning a Kenyan *Cowdria* isolate (Kocan *et al., 1*987b). Three species of *Amblyomma* are most frequently observed in Kenya, *A. variegetum, A. gemma* and *A. lepidum,* (Walker, 1974). *A. variegatum* is widely distributed and can occur up to 8,500 feet above sea level (Walker, 1974). However there has been virtual eradication of *Amblyomma* in many high potential areas, where acaricides have been used intensively in the prevention of East Coast fever (ECF). The Kenyan livestock population, estimated at about 12 million cattle and over 15 million sheep

and goats (Kariuki, 1992) is believed to all be at risk or potential risk of heartwater.

Heartwater is not a notifiable disease and clinical cases occur sporadically. Overshadowed by ECF, and coupled with the difficulty of diagnosis, clinical and post mortem, most cases go unreported even with experienced staff (Kocan *et al.*, (1987b).

With the advent of immunisation against ECF, farmers have reduced the frequency of acaricide application (Kariuki *et al.*, 1994) in favour of strategic control (Uilenberg, 1984; Purnell, 1984). Heartwater, therefore is likely to re-establish in ECF immunisation areas through movement of carrier stock having *Amblyomma* ticks (Norval, 1979; Lawrence *et al.*, 1980; Mutugi, 1983). Before heartwater is established endemically, susceptible livestock are likely to suffer outbreaks of heartwater disease, bringing about the need to put heartwater control measures into place.

This study focused on isolation of *Cowdria ruminantium* from different geographic locations in the country. They were characterised *in vivo* to test their infectivity and pathogenicity in sheep and mice, and *in vitro* using molecular techniques. A laboratory cross immunity trial was carried out in sheep using selected mild and virulent isolates to assess the immunising and cross protective potential. A one way cross immunity trial was attempted in mice. Finally, the susceptibility of the two main Kenyan vectors was attempted for two of the isolates. The findings were put into perspective of the tick-borne diseases situation, and in particular heartwater disease in Kenya.

The objectives of this study were:-

a) To isolate *Cowdria ruminantium* from different geographic locations in Kenya

b) To characterise the *Cowdria* isolates by *in vivo* and *in vitro* methods

   i) To study the infection due to needle inoculation of susceptible Corriedale sheep.

   ii) To do one and two way laboratory cross immunity trials to assess the cross protection of the isolates in Corriedale sheep.

iii) To study the infection and cross protection (with selected isolates), in Balb/C mice.

iv) To study the susceptibility of the two main species of *Amblyomma* in Kenya to two selected Kenyan *Cowdria* isolates.

v) To tentatively identify a possible vaccine candidate for attempting to control the disease with in this country.

CHAPTER ONE

LITERATURE REVIEW

1.1 General background

Heartwater is an acute, febrile, septicaemic, infectious, noncontageous but inoculable disease of domestic and wild ruminants which is accompanied by nervous, pulmonary and gastro-intestinal disorders (Henning, 1956; Uilenberg, 1981; Mebus and Logan, 1988). The causal agent *Cowdria ruminantium* is a rickettsia transmitted by a number of ixodid ticks of the genus *Amblyomma (*Henning, 1956).

The disease occurs in most of subsaharan Africa, Madagascar and some tropical islands in the Caribbean (Camus and Barré, 1982; Provost and Bezuidenhout, 1987). The disease affects cattle, sheep, goats and wild ruminants (Henning, 1956; Mebus, and Logan, 1988). The disease has been disregarded for a long time until recent advances in diagnosis (Jongejan, 1991a), have made it possible to study epidemiological aspects. Unequivocal diagnosis of heartwater is only possible by the microscopic demonstration of rickettsial colonies in endothelial cells of blood vessels in smears of the brain cortex (Uilenberg, 1981). Control of the disease is achieved by either regular acaricide application or by 'infection and treatment' immunisation which can be rather unsatisfactory (Uilenberg, 1981). Oxytetracyclines are the drugs of choice for treatment, but chemotherapy often fails due to the rapidity of the disease and the prognosis is poor in advanced cases (Uilenberg, 1981).

The importance of heartwater is accounted for by its second rank only to theileriosis (ECF): two major tick borne diseases that cause constraints to the African livestock development and production (Uilenberg, 1981). There are no accurate economic evaluations of losses caused by heartwater, but estimates are thought to run into millions of dollars per anum for any one country where it occurs (Camus *et al.,* 1996).

A number of authors have reviewed the literature (Alexander, 1931; Curasson, 1943; Haig, 1955; Henning, 1956; Neitz, 1968; Andreasen, 1974; Ilemobade, 1976; Uilenberg, 1971, 1977; Ramisse and Uilenberg, 1971; Camus *et al.,* 1996).

1.2 Aetiology

The causative agent of heartwater is the rickettsia *Cowdria ruminantium* (Cowdry, 1925). It was first shown to be a transmissible agent by Dixon (1898), and Edington (1898), when they were able to reproduce the disease in a susceptible animal by intravenous inoculation of blood from an infected animal. Lounsbury (1900), was the first to show it had a vector: *Amblyomma hebraeum*. But it was Cowdry (1925a, 1925b), who first conceived the organism to be a rickettsia and elucidated it in the tissues of the host and the vector. Cowdry named the organism *Rickettsia ruminantium* because of the way this Gram negative staining, intracellular bacterium, was transmitted by an arthropod. Moshkovski (1947), renamed it *Cowdria ruminantium* in honour of Cowdry, after he proposed it as a subgenus (Moshkovski, 1945), because it differed from other rickettsiae in that it could not be made to infect laboratory animals or grow *in vitro*.

Cowdria is a rickettsia and a member of Proteobacteria belonging to the alpha or gamma groups which share a number of phenotypic as well as molecular characteristics (Drancourt, and Raoult, 1994). Pienaar (1970) found striking, the morphological resemblance between *Cowdria* and *Chlamydia*. Jongejan *et al.*, (1991a), and Jongejan *et al.*, (1991b) found a monoclonal antibody (Mab) directed at the major outer membrane protein of *Chlamydia* recognised a *Cowdria* antigen. But Van Vliet *et al.* (1992), comparing the 16S ribosomal DNA found there was a closer genetic relationship between *Cowdria* and *Ehrlichia* than between *Cowdria* and *Chlamydia*. The close relationship of *Cowdria* has also been found in serologic cross reactivity with a number of *Ehrlichia* species such as *E canis,* and *E equi* (Logan *et al.*, 1986; Holland *et al.*, 1987), and *E bovis, E phagocytophila* and *E ovina* (Du Plessis *et al.*, 1987). Williams and Vodkins, (1987), found that *Cowdria* and *Ehrlichia* had cell tropisms that were very similar. Van Vliet *et al.*, (1992) and Dame *et al.*, (1992), found *Cowdria* had a close relationship with *Anaplasma* on the 16s ribosomal DNA.

1.2.1 CLASSIFICATION

According to Bergey's Manual of systematic Bacteriology (Weiss and Moulder, 1984) *Cowdria ruminantium* (Cowdry, 1925) belongs to the Order *Rickettsiales,* Family *Rickettsiaceae,* Tribe *Ehrlichieae* which contains three genera, *Ehrlichia, Cowdria* and *Neorickettsia.*

1.2.2 LIFE CYCLE

Briefly, the life cycle involves infection of the tick when the organism is ingested with blood of the host as the tick feeds. The *Cowdria* organism invades the tick gut epithelium where it multiplies and invades other organelles including salivary glands. They are inoculated into a new host when the tick feeds in the next instar. In the ruminant host the organism infects endothelial cells lining blood vessels where it multiplies and disrupts the infected cells. The organisms are released into circulation reinvade endothelial cells and possibly other cells or are picked up by a feeding tick.

It has been possible to trace the development of *Cowdria ruminantium* only in part in the ruminant host and to a greater extent in the tick vector. Study of the morphology and development of *Cowdria ruminantium* in the tick vector and host using various techniques has led to certain deductions about its life cycle.

The realisation by Cowdry that heartwater is caused by a rickettsia found in the tissues of the host and the tick vector (Cowdry, 1925a & b, 1926a & b), provided a basis for further studies on the organism. Cowdry (1925a), observed *Cowdria* in the culminate stages of development: in the capillary endothelium of renal glomeruli, superficial grey matter of the cerebral cortex and various other organs but not some organs such as the lung or liver.

Other workers have since observed the organism in various organs or tissues of the host and vector which suggest its transport and progression in the different stages of its life cycle. It has been seen in macrophages, monocytes, endothelial cells of renal tubules and the brain capillaries, and blood leukocytes in the vertebrate host. It has been observed in the gut epithelium, malpigian tubules, salivary glands and other organelles in the invertebrate host.

After inoculation by the tick, *Cowdria* is thought to initially develop in reticulo-endothelial cells of lymph nodes before invading endothelial cells of blood vessels (Du Plessis, 1970). Du Plessis (1975), and Ilemobade (1976), found the organisms in reticulo-endothelial cells and in peritoneal macrophages, while Pienaar (1970), saw it in monocytes. It can be hypothesised that from a localised development, the organism is transported to the rest of the body via the lymphatics and blood which, although difficult to detect microscopically, was first demonstrated by Dixon (1898) and Edington (1898), by transfusing blood and transmitting the disease from an infected animal to a susceptible one. Extracellularly *Cowdria* has been demonstrated in plasma by culture (Byrom *et al.*, 1991), and subinoculation of plasma (Ilemobade and Blotkamp, 1978). Thereafter, the organism appears to invade a number of cells and has been shown to be in different fractions of blood and blood cells (and to be infective) (Ilemobade, 1976, Fawi and Karrar, 1960), neutrophilic granulocytes by culture (Logan *et al.*, 1987; and Sahu *et al.*, 1983), and animal inoculation of cultured granulocytes (Jongejan *et al.*, 1989; Logan, 1987).

In the tick, colonies of the organism have been demonstrated in gut, salivary gland cells, haemocytes and Malpighian tubules of infected *Amblyomma* ticks (Kocan, and Bezuidenhout, 1987; Kocan *et al.*, 1987a; Bezuidenhout, 1984; Prozesky *et al.*, 1986; Kocan *et al.*, 1987b), and could be followed in their development and invasion of new organelles. It was conceived that a developmental cycle of the organism occurs in its invertebrate host, of which the main mode of multiplication appeared to be binary fission. *Cowdria* has also been demonstrated in different organelles (salivary glands and guts) by PCR (Kocan and Bezuidenhout, 1987). Jongejan *et al.*, (1991c), observed that *Cowdria* had a *Chlamydia*-like growth cycle in tissue culture.

1.2.3 MORPHOLOGY

Cowdria occurs in close-packed colonies in endothelial cells of blood vessels in the host (and in vector) tissues. It forms large and sometimes small clumps or colonies within a cytoplasmic vacuole usually situated to one side of the nucleus of the host cell. The colony size varies because the number of individual organisms (seen as granules under the light microscope) in a colony can number from less than 10 to

several hundreds (Uilenberg, 1981). Individual organisms may have different shapes ranging from coccoid to bacillary, and forms from densely solid to open rings. Under the light microscope the smaller bodies appear coccoid while larger ones may be ring, rod horse shoe or irregular masses (Uilenberg, 1981).

Electron microscopy has made it possible to observe the ultrastructure of *Cowdria* which is very similar in both the vertebrate host and the tick vector, Pienaar (1970); Prozesky and Du Plessis (1987); Hart *et al.*, 1991); Kocan *et al.*, (1987a); Kocan *et al,*. (1987b); Totté *et al.*, (1993a); Martinez *et al.*, (1993b); Prozesky *et al.*, 1986). The rickettsia in one colony are usually of one size but individual diameter vary from one colony to another being from 0.2μm to 2.5μm. Prozesky *et al.*, (1986), have identified elementary, reticulate, and intermediate bodies of the organisms in tissue culture. There was no significant ultrastructural difference between *Cowdria* from brain and ticks, apart from the presence of a single defined electron-transparent region in brain isolates while organisms in ticks had several electron transparent areas (Neitz, *et al.*, 1986a).

1.2.4 ANTIGENIC AND MOLECULAR CHARACTERISATION

Antisera raised to *C ruminantium* have been shown to recognise a number of antigens (Rossouw *et al.*, 1990). However, only the immunodominant ones have been of interest and studied in detail. These include the 32 kilodalton (kDa) protein antigen (Jongejan and Thielemans, 1989) which was also called Cr32. Since its molecular weight varies between isolates it was re-named the Major Antigenic Protein (MAP1) (Barbet *et al.*, 1994). This may be because the genes encoding the MAP1 protein from *Cowdria ruminantium* isolates obtained from different geographical areas have sequence heterogeneity, as shown by Reddy *et al.* (1996). Immunological tests based on the MAP1 antigen have been developed and utilised in serodiagnosis (Jongejan *et al.*, 1991d; Kock *et al.*, 1993; Kobold *et al.*, 1992; Du Plessis *et al.*, 1993). The antigens that have been identified include 21, 32, 40, 46, 58, 85 and 160 kDa (Mahan *et al.*, 1994b).

Large quantities of *Cowdria* DNA that is relatively free from host cell DNA has become available due to *in vitro* culture which in turn, has made DNA cloning

experiments possible. The first experiments involved cloning fragments specific for *Cowdria* DNA from which a probe, the pCS20, that hybridised only with *Cowdria* DNA was developed (Waghela *et al.*, 1991; Mahan *et al.*, 1992). By targeting specific genes, the gene encoding the MAP1 protein was cloned and characterised (Van Vliet *et al.*, 1994) as was the gene encoding the 21 kDa protein antigen (Mahan *et al.*, 1994b), the 16S rRNA gene (Van Vliet *et al.*, 1992) and *C ruminantium* homologues of the *E. coli* heat shock proteins GroE (Lally *et al.*, 1995). All serological tests developed so far have had specificity problems due to cross reactions with *Ehrlichia* and attempts have been made to rectify this with better success (Kock *et al.*, 1995.; Van Vliet *et al.*, 1995; Katz *et al.*, 1996). Using the obtained sequences, attempts were made to develop more specific serological tests using recombinant antigen (Van Vliet *et al.*, 1995; Katz *et al.*, 1996), but cross reactivity with some ehlichial antigens remains.

The examination of the 16S rRNA sequence has shown that the *Cowdria* sequence is much more similar to that of a number of *Ehrlichia* (Van Vliet *et al.*, 1992) and also *Anaplasma (*Dame *et al.*, 1992) than with the other rickettsial agents.

1.3 The disease

1.3.1 INCUBATION

According to Neitz (1968), the incubation in cattle averages around 18 days (following tick transmission) but ranges between 1-3 weeks after intravenous inoculation. It is shorter in small ruminants averaging 9-10 days but varying from 5 - 35 days (Alexander, 1931, Yunker, 1996). This period is longer in naturally acquired infection because of the 'pretransmission period' of tick feeding.

1.3.2 CLINICAL SIGNS

The clinical signs have been reviewed by Van de Pypekamp and Prozesky (1987), and depend on species, age, immune status, virulence of the *Cowdria* stock and route of infection. The disease usually starts with a sudden rise in body temperature that commonly exceeds 41°C and is not accompanied by any other sign and may therefore be missed. Fever usually remains high but may drop just before death. Gradually other

signs appear and include listlessness, tachypnoea, loss of appetite, nervous signs and sometimes diarrhoea. Other signs that may be seen include incoordination, muscular tremor, lip licking and death.

The course of infection varies from inapparent to peracute. The inapparent and mild forms occur in cattle of all ages in endemic areas, partially immune animals and in the very young of all breeds of affected ruminants. Symptoms that occur are slight and may go unnoticed comprising increase in respiration and listlessness followed by recovery. At the other extreme peracute cases occur in exotic breeds of cattle sheep and goats (Van de Pypekamp and Prozesky, 1987). An animal usually looks normal but may collapse suddenly, convulse and die. Most susceptible animals suffer the acute form of the disease which starts with a high fever and is accompanied by increased respiration, nervous disorder and sometimes intestinal disorders. The disease lasts a variable number of days and the animal may show a combination of clinical symptoms including circling, twitching eyelids, licking lips, staggering, abnormal posture, teeth grinding, and hyperaesthesia. Finally the animal may collapse, make paddling movements, may have nystagmus, opisthotonus and chewing movements. There may be several convulsive attacks before death (Alexander, 1931). Intestinal symptoms include a profuse fetid diarrhoea which may be haemorrhagic. Sub acute disease has similar symptoms but they are less marked. The disease usually lasts longer (for about a week), after which there may be death or gradual recovery (Van de Pypekamp and Prozesky, 1987). Pregnant animals may abort (Alexander, 1931; Neitz, 1968; Camus *et al.*, 1996). The prognosis is usually grave for the peracute and acute forms of the disease and usually poor even in subacute forms (Camus *et al.*, 1996).

1.3.3 PATHOLOGY

Autopsy lesions have been studied in depth and been found to be variable (Steck, 1928; Alexander, 1931). They have also been reviewed and augmented by Uilenberg (1983) and Prozesky (1987a). Post mortem lesions are similar in all species and are characterised by marked effusion of fluid into body cavities (Prozesky, 1987a). There is commonly hydropericardium (from which the disease derives its name),

hydrothorax, and sometimes ascites. The lungs, mediastinum and associated lymph nodes may be oedematous and the bronchi and trachea have some froth (Yunker, 1996). Moderate spleenomegally was reported in most small ruminants by Steck (1928), but Uilenberg (1971), and Prozesky (1987a), did not encounter spleenomegally frequently. Other findings included moderate swelling of lymph nodes and pallor of kidneys while the liver had mild congestion and fatty degeneration (Neitz, 1968; Prozesky, 1987a). Enteritis is said to be less frequent in small stock while the macroscopic lesions of the brain are limited to meningeal oedema and congestion of the meningeal blood vessels. (Clark, 1962; Prozesky and du Plessis, 1984).

The liver is usually engorged and various degrees of gall bladder distension have been documented by various researchers (Alexander,, 1931; Henning, 1956; Andreasen, 1974; Uilenberg, 1971; Ilemobade, 1976).

1.3.4 PATHOGENESIS

The Pathogenesis of heartwater is poorly understood (Uilenberg, 1981; Du Plessis *et al.*, 1987b). The presence of the organisms does not seem to grossly affect infected cells. Nervous symptoms appear to be more severe than can be explained by the severity of the brain lesions. Lung oedema is only sometimes severe enough to have caused death by asphyxiation. However, effusion of fluid from the vascular system probably lead to a fall in blood pressure and may contribute to death.

Destruction of the endothelial cells of blood vessels leading to increased permeability to fluids and subsequent effusion into body tissues and cavities (Clark, 1962; Prozesky and du Plessis, 1984), seems to be the chief detrimental event. However the factors that cause this destruction remain obscure and speculations continues as to how this comes about.

Renal ischaemia appeared to be the cause of kidney lesions seen in Angora goats, Prozesky and Du-Plessis (1985). An endotoxin of short duration has been reported and its presence associated with severe clinical signs (rapid and laboured breathing, cyanosis and recumbency) (Van Amstel *et al.*, 1988, 1994), in fatal cowdriosis in sheep. Evidence of other studies point to pathogenesis of the disease being the result

of a hypersensitivity reaction (Du Plessis *et al.*, 1987b), beginning with the appearance of antibodies in the immune reaction (Langa, 1995).

Various biochemical and haematological changes which appear to be consequences rather than the cause have been observed. A severe drop in serum protein, especially in the albumin levels (Van Amstel *et al.*, 1988), is attributed to the increased capillary permeability as evident from the fact that protein content of the pericardial fluid was approximately that of the serum in a calf which died. The osmolarity of the effused fluid was higher than that of the blood (Van Amstel *et al.*, 1988), which would account for fluid being drawn out of the vascular system. Changes in serum electrolyte levels were insignificant except the total calcium levels which tended to decrease to below normal during the acute stage of the disease (Van Amstel *et al.*, 1988). Progressive anaemia and fluctuations in the total and differential white cell count: eosinopenia and a lymphocytosis were reported (Van Amstel *et al.*, 1988).

1.4 Diagnosis

A tentative diagnosis of heartwater is made on case history (tick exposure), the clinical signs (high fever and nervous signs), and on the post mortem lesions (having hydrothorax and hydropericardium). These must be differentiated from other conditions that cause nervousness and fluid in body cavities. A diagnosis of heartwater is confirmed only by identifying *Cowdria* organisms in an infected, (Synge, 1978) or dead, (Purchase, 1945) animals. Observing morulae in the cytoplasm of cultured neutrophils separated from the blood of a febrile animal (Logan *et al.*, 1987) can be used to confirm a diagnosis of heartwater. This technique may not be that useful in the field as it takes 24 to 72 hours. Detection of *Cowdria* by PCR of DNA extracted from the blood or the neutrophil cultures with *Cowdria* specific primers (Mahan *et al.*, 1992; Kock *et al.*, 1995), is a more convincing diagnosis. The burden of proof still remains with showing *Cowdria* colonies in endothelial cells of the brain to make unequivocal diagnosis. Heartwater occurring for the first time in a locality therefore, must be confirmed by subinoculation of blood from the suspect case and the organisms demonstrated in the brain crush smear of a recipient susceptible host (Camus and Barré, 1987; Van Vliet, 1995). The organisms may be identified by fixing

thin sections or brain squash smears in alcohol, staining with Giemsa and finding typical azurophilic colonies in the endothelial cells of capillaries under the light microscope. An immunohistochemical staining of sections with specific monoclonal antibody (Jardine *et al.*, 1996), appears to give good results. Stains other than Giemsa (Camus *et al.*, 1996), have become popular (such as Diff Quick) for their expedience in certain laboratories. Inoculation of infective material into endothelial culture may also yield positive *Cowdria* culture, but this method is fastidious and time consuming.

1.4.1 DIFFERENTIAL DIAGNOSIS

The lack of pathognomonic signs of heartwater disease in the field require that a tentative diagnosis be arrived at before a confirmation is made. Consideration of the local disease situation and the herd history is taken into account (Camus *et al.*, 1996).

The predominant signs observed may be nervous symptoms which simulate diseases such as rabies, strychnine poisoning, tetanus, and cerebral babesiosis. Haemorrhagic diarrhoea may resemble rinderpest, coccidiosis or anthrax (Camus *et al.*, 1996).

1.4.2 SEROLOGICAL DIAGNOSIS

Good serologic tests are necessary for an accurate assessment of the distribution and epidemiology of heartwater. Yet, development of serologic assays has made slow progress due to the difficulty of obtaining sufficient quantities of good quality antigen. It was not until endothelial cultures were successfully established (Bezuidenhout *et al.*, 1985) that the situation completely changed.

The first serologic test was a capillary flocculation test developed by Ilemobade and Blotkamp (1976), using antigen extracted from the brain of infected animals. A compliment fixation test was developed by Du Plessis (1982) quoted by Jongejan (1991a), and (Musisi and Hussein, 1984), also using acetone extracted antigen from the brain. However, these early tests had low sensitivity and specificity and therefore detected antibody (most likely peak levels only) for relatively short periods.

Based on the mouse pathogenic Kumm stock (Du Plessis and Kumm, 1971), Du Plessis (1981b), developed an IFA test using the infected mouse peritoneal macrophages as antigen. This test was useful in that positive titres were detected for

many months (Du Plessis, 1984b; Du Plessis and Malan, 1987a; Camus, 1987), however, there was difficulty of producing sufficient macrophages.

An enzyme linked immunosorbent assay (ELISA) that utilised antigen isolated from sheep brain or infected *A hebraeum* by affinity chromatography (Viljoen *et al.*, 1985, Neitz *et al.*, 1986b, 1986c), and percoll gradient centrifugation, Neitz (1986a) all had the similar problem of not producing sufficient antigen for large scale use.

Neutrophil cultures as a source of antigen (Logan *et al.*, 1987), for an IFAT (Logan, 1987; Jongejan *et al.*, 1989), was promising in that antigen could be obtained from any isolate. However it was limited by the variability in the percent infected cells in the cultures, some failing to produce any infected neutrophils. It was found necessary to reduce non specific fluorescence (Jongejan *et al.*, 1989) with this test. It was found less useful because it was serotype sensitive for heterologous stocks (Jongejan *et al.*, 1988, 1991b).

Endothelial cell culture (Bezuidenhout *et al.*, 1985), has overcome the problems for serologic assays by producing large quantities of *Cowdria* elementary bodies (EBs) free of host cell components (Bezuidenhout, 1987b). Based on tissue culture EBs antigen a superior IFA test with strong fluorescence was developed (Martinez *et al.*, 1990; Du Plessis *et al.*, 1993), that detected all serotypes equally.

The occasion was taken to develop ELISA tests, such as the monoclonal antibody mediated competitive ELISA (Jongejan *et al.*, 1991d), and indirect ELISA (Martinez *et al.*, 1993), which are capable of testing large numbers of samples. The simultaneous tests (Martinez *et al.*, 1990; Du Plessis *et al.*, 1993), had good sensitivity but extensive cross reaction with putative *Ehrlichia* was present. An immunoblotting technique for diagnosis was also developed (Mahan *et al.*, 1993a). The old problem in the tests with improved antigen remained in the serological cross-reactivity with *Ehrlichia* agents that was first detected by Logan *et al.* (1986) and persisted in all tests that have been developed so far (Kobold *et al.*, 1992; Camus *et al.*, 1993; Jongejan *et al.*, 1993c; De Vries *et al.*, 1993; Kelly *et al.*, 1994; Van Vliet, 1995) and still remains to be solved to some extent. This is important in sero-epidemilogical surveys as demonstrated by Martinez *et al.* (1992, 1993), and De Vries *et al.*, 1993).

Inspite of this serologic tests remain valuable tools for monitoring experimental animals.

1.5 Epidemiology

Heartwater is a disease of domestic and wild ruminants (Mebus, and Logan, 1988). There are three situations with regard to heartwater: endemically stable, unstable and disease free. Endemic stability exists with a high disease transmission beneath apparent quiescence of clinical cases (e.g. Deem *et al.*, 1996c); a situation of endemic instability (characterised by epidemics or outbreaks), can arise from an endemically stable state for a number reasons. For example, outbreaks involve susceptible animals becoming exposed to the disease by moving into an endemic environment (Macpherson, 1995). The disease may also be brought into a 'clean' environment by animals and/or vector ticks having the disease (Perreau *et al.*, 1980; Camus and Barré, 1990). For the former situation to evolve into persistent presence of infection vector ticks must already exist in the environment of the susceptible animals (Barré *et al.*, 1987). Usually, the presence of heartwater is first recognised after introduction of susceptible animals into an endemic environment such as happened when European stocks were first introduced into South Africa, or when farmers in African countries have tried to upgrade their dairy cattle by importing exotic cattle or their crosses (Uilenberg, 1983).

Stability is brought about by early infection of young animals during the period of innate resistance, leading to immunity, development of carriers, and immunity elevated in the herd by constant low tick challenge of the whole population.

There is evidence that heartwater is endemically stable in much of sub saharan Africa (Howell *et al.*, 1981; Bezuidenhout, 1985; Bezuidenhout and Bilgalke, 1985; Norval *et al.*, 1992). It is maintained by domestic ruminants alone (as happens in Madagascar), or in the presence of wild African ruminants (Uilenberg, 1981; Kock *et al.*, 1995). Yet scrutiny of the known facts about heartwater, could not explain satisfactorily how young animals become infected early enough in life to acquire immunity. The following are observations made on heartwater. Ticks are most readily infected by clinical cases to produce high tick infection rates (Barré and Camus, 1987;

Waghela *et al.*, 1991; Yunker *et al.*, 1993; Peter *et al.*, 1993). The frequency of clinical heartwater in endemic areas is low therefore, a high infection rate in ticks is similarly infrequently injected into the environment. Carrier state of an intermittent nature exists among ruminant hosts (Andrew and Norval, 1989; Camus, 1992) and ticks feeding on post recovery (carrier) animals acquire no or very low infection rates (Barré and Camus, 1987; Peter *et al.*, 1993). As field ticks have a limited opportunity of becoming infected they usually have and would be expected to acquire mostly low infection rates (Camus, 1987, Camus and Barré, 1987, Du Plessis, 1985). Exceptionally, high infection rates have been found (Norval *et al.*, 1990; Du Plessis and Malan, 1987c; Gueye *et al.*, 1993). (The infection rate from other reservoir hosts has not been examined, but is also thought to be low, although high infection rates with *Theileria parva* have been reported to occur in *Rhipicephalus appendiculatus* ticks fed on carrier buffalo (Grootenhuis *et al.*, 1987; Ochanda, 1994)). Most young animals appear not to acquire tick burdens until they are 3 to 4 months of age (Du Plessis *et al.*, 1992b; Norval *et al.*, 1995). Young ruminants posses an innate resistance not related to the immune status of the dam (but which may be affected by it, Deem *et al.*, 1996b), However, it is lost within three to four weeks (Neitz and Alexander, 1941; Neitz *et al.*, 1947; Alexander *et al.*, 1946; Uilenberg, 1981), leaving the animal fully susceptible to disease. It follows that calves do not acquire tick derived *Cowdria* infection until after innate resistance has waned. There should be a high incidence of disease prevailing after this period but most calves in endemic areas develop only mild or inapparent infection on challenge (Du Plessis *et al.*, 1984; Du Plessis and Malan, 1987b; Deem *et al.*, 1996b).

Under these circumstances, the necessary reservoir of infection maintaining and driving endemic stability could not be explained satisfactorily until, Deem *et al.* (1996a), demonstrated an additional source of infection for the calves: verticle transmission. This unique mode of transmission occurs from a dam to its calf perinatally in cattle in endemic areas, but the extent of its occurrence is not yet known. Deem *et al.* (1996c), hypothesised that calves are initially infected with *C ruminantium* before they are infested with *Amblyomma* ticks (thus acquiring resistance to clinical heartwater infection that lasts beyond the three to four weeks

(sole innate resistance)).Vertical transmission would appear to ensure that a calf is exposed to *Cowdria* early in life for it (with the help of maternal antibodies) to acquire immunity while still possessing innate resistance and making it immune to subsequent tick challenge (Deem *et al.*, 1996c).

On the other hand, endemic instability occurs if transmission does not take place early enough in life due to implementation of tick control or low vector (low population) brought about by seasonality of the conditions being marginal for tick survival, that individual animals escape infection for several months (Alexander, 1931; Bonsma, 1944)). The growing animal looses innate resistance and becomes susceptible, the level of immunity in the herd falls and animals succumb to disease when challenged (usually in epidemics). (These events may not occur in a resistant population).

Heartwater can come to susceptible livestock from a number of sources. Infected vectors which are long lived and act as reservoirs of infection in their own right (Ilemobade, 1976; Norval *et al.*, 1992), and vertebrate carrier host (Oberem and Bezuidenhout, 1987). Transhumance (Macpherson, 1995), or shipment, of animals from a heartwater free into an endemic area can lead to outbreaks (Norval and Lawrence, 1979; Logan *et al.*, 1988; Gueye *et al.*, 1984). Infected vectors can be spread on cattle, wildlife and non-ruminant hosts of *Amblyomma* to new areas (Hoogstral, 1961; Perreau *et al.*, 1980; Wilson and Richard, 1984; Barré *et al.*, 1987; Petney and Horak, 1988; Matton and Van Melckebeke, 1989). The Caribbean focus now poses a serious threat for livestock on the American mainland (Uilenberg, 1982; Barré *et al.*, 1987; Uilenberg, 1990). Redistribution of infected ticks has occurred from neighbouring endemic areas after disintegration of intensive tick control (Lawrence *et al.*, 1980; Norval *et al.*, 1991). The receding of favourable conditions in and out of an ecologically marginal area may lead to corresponding disease appearance and disappearance (Norval *et al.*, 1992). It would appear that raising highly susceptible stock in an endemic area does not readily establish endemic stability and 'small epizootics or sporadic outbreaks' have been observed (Camus and Barré, 1987).

1.5.1 VECTORS

The only known natural vectors of *C ruminantium* are ticks of the genus *Amblyomma* (*Acari: Ixodidae*) (Bezuidenhout, 1987). The known African *Amblyomma* vectors include ten species: *A. variegatum, A. hebraeum, A. pomposum, A. gemma, A. lepidum, A. cohaerens, A. tholloni, A. sparsum A. astrion* and *A. marmoreum* (Walker and Olwage, 1987). Three American *Amblyomma* have transmitted heartwater experimentally, *A. cajannense* (Uilenberg, 1983) *A maculatum* (Uilenberg, 1982), and *A. dissimile* (Jongejan, 1992).

The importance of different *Amblyomma* species depends on the extent of their adaptation to domestic livestock and their efficacy as vectors, and ecological factors (Bezuidenhout, 1987). Availability of certain hosts limit their distribution due to host preference of the adults ticks. This is seen in the limited distribution of certain ticks such as *A. tholloni* the elephant tick. *A hebraeum* is the most important vector in Southern Africa as it has a wide host range and feeds well on domestic livestock in all its instars. It may also feed on wild animals and rodents (Petney *et al.*, 1987). *A variegatum* feeds well on cattle, and infests sheep and goats less (Hoogstraal, 1956). It is the most widespread vector on the African continent and its surrounding islands. *A variegatum* has also been transported to and established in the Caribbean islands (Camus, 1987), and has the potential to become established on the American mainland (Sutherst and Maywald, 1985).

The other *Amblyomma* vectors are known to be major vectors regionally; *A. lepidum*, has been involved in field outbreaks of heartwater in the Sudan as has *A. astrion* on the islands of Sao Tome and Principe, and *A. pomposum* in Angola (Uilenberg, 1983).

1.5.2 HOST RANGE

Cowdria has a wide host range (Oberem and Bezuidenhout, 1987a; Uilenberg, 1983; Camus *et al.*, 1996). However, it is mainly domestic ruminants, cattle, sheep and goats that suffer clinical disease. Other hosts include wild African ruminants (e.g. antelopes, buffalo, eland, water buck, bush buck, and giraffe), birds (guinea fowl), scrub hare and the tortoise (Oberem and Bezuidenhout, 1987a; Uilenberg, 1983). Non

African ruminants, some of which are wild have also been found to be susceptible to disease and may die from it (Oberem and Bezuidenhout, 1987a; Camus *et al.*, 1996). These include the Indian buffalo and various members of the deer family. Most wild animals do not show any clinical signs. Some wild rodents have been infected experimentally (Camus *et al.*, 1996), and laboratory mice are susceptible to some strains of *Cowdria* (MacKenzie, and Van Rooyen, 1981; McHardy, and MacKenzie, 1984; MacKenzie and McHardy, 1987).

1.5.3 DISTRIBUTION

Heartwater distribution is thought to closely match that of the vector tick distribution (Provost and Bezuidenhout, 1987; Uilenberg and Camus. 1993). This includes Africa south of the Sahara, most islands around the continent and some Caribbean islands. Except in the desert and cold highlands, where conditions do not favour their survival, *Amblyomma* ticks are widely distributed in Sub Saharan Africa (Walker and Olwage, 1987). Therefore, heartwater is thought to occur in all Southern African countries except Namibia, which is too dry to support *Amblyomma* species (Purnell, 1984). From Cape province to Kassala in the Sudan and from Senegal in West Africa to Somalia in East Africa (Uilenberg, 1983). Presence of heartwater has been reported and confirmed in at least 43 African countries from the turn of the century (Tritchard, 1838 in Neitz, 1968), to the early nineties (Boorder *et al.*, 1993) quoted by (Camus *et al.*, 1996). In Kenya, it was first reported by Daubney (1929-1930).

Apart from Africa, the disease has been reported in the Caribbean islands of Guadeloupe and Antigua, and Marie Galante (Perreau *et al.*, 1980; Camus, 1987; Camus and Barré, 1987, 1988). There is concern over the possibility of it reaching the susceptible ruminants on the American mainland (Barré *et al.*, 1987).

1.5.4 TRANSMISSION

1.5.4.1 ARTIFICIAL TRANSMISSION

Artificial transmission has been achieved by needle i/v inoculation of both infected vector (invertebrate) extract (Neitz and Alexander, 1945; Birnie *et al.*, 1985; Camus and Barré, 1987; Prozesky, 1987a; Du Plessis, 1985), and infected host (vertebrate), tissues (Ilemobade, 1976; MacKenzie and Van Rooyen, 1981; Byrom *et al.*, 1993).

The establishment of endothelial tissue culture (Bezuidenhout *et al.*, 1985), has brought about the use of cultured elementary bodies to induce heartwater infection (Jongejan *et al.*, 1989, 1991). Infected neutrophils from culture have also induced heartwater when inoculated i/v to a susceptible host (Jongejan *et al.*, 1989; Logan, 1987).

1.5.4.2 NATURAL TRANSMISSION

Natural transmission of heartwater has been associated only with the species of *Amblyomma* ticks. *Amblyomma* are three host ticks (each instar dropping off after feeding on each host) and spending long interstadial periods in the vegetation between feeds on hosts (Jongejan and Uilenberg, 1994). *C. ruminantium* is transmitted transtadially (Bezuidenhout, 1987a), but transovarial transmission has also been reported once only (Bezuidenhout and Jacobz, 1986). Because of this, it is expected that *C ruminantium* is picked up by larvae, nymphs and adults but transmitted only by the nymphs and adults. It follows that unfed larvae are the only instar free of infection and transmit infection only after becoming nymphs (Bezuidenhout, 1987a). Once infected a tick remains infected in all subsequent instars, whether it feeds on a susceptible species of animal in the following instar or an insusceptible one. Intrastadial transmission has caused infection to susceptible animals experimentally (Norval *et al.*, 1990; Kocan *et al.*, 1993), and is thought to occur when *Amblyomma* ticks quickly leave a dead host and find a new one. Larvae which feed on carrier small mammals, birds, or reptiles may acquire infection, if they feed on non carrier or insusceptible hosts larvae remain uninfected. Nymphs are usually the to first acquires the infection and transmit it as adults. Ticks usually feed for a variable period before transmission occurs but Neitz (1968), found this period was frequently less than 24 hours.

Transmission has now been shown to also occur from a dam to its calf perinatally without the aid of a tick vector (Deem *et al.*, 1996a). The extent to which this unique transmission occurs, has not been fully investigated.

1.5.5 SUSCEPTIBILITY

The variability of outcome on infection with *Cowdria* has made researchers realise that different animals are not equally susceptible (Uilenberg, 1983; Camus *et al.*, 1996). Susceptibility has been found to be determined by a number of factors some of which are intrinsic and some extrinsic. Non- indigenous (exotic), breeds of cattle sheep and goats have been found to be more susceptible than indigenous breeds (Smith, 1930; Lounsbury, 1904, 1905; Curasson, 1932; Neitz and Alexander, 1941; Rousselot, 1953; Bonsma, 1944; Sutton, 1960; Van Der Merwe, 1979; Du Plessis *et al.*, 1994). But indigenous animals from non endemic areas were found to be as susceptible as exotic animals (Ilemobade, 1977; Gueye *et al.*, 1989). It was suggested that in some instances, resistance to *Cowdria* could be inherited by natural selection (Neitz and Alexander, 1945; Uilenberg, 1981). For example, Creole goats appear to have a genetically controlled resistance (Matheron *et al.*, 1987, 1991). Creole Zebu also do not experience clinical heartwater (Camus, 1987). Some wild ruminants (spring buck and eland), and non African ruminants (Asian buffalo, deers), have been found to be susceptible to heartwater (Camus *et al.*, 1996), while other ruminants are refractory or difficult to infect. There remains some doubt about the camel (Karrar, 1960). Okewole *et al.* (1993) diagnosed heartwater in a dead African elephant. The leopard tortoise and guinea fowl (Bezuidenhout and Olivier, 1986), and scrub hare (Bezuidenhout, 1990) were found to become infected subclinically but may be important only as reservoirs of infection for *Amblyomma* ticks.

1.5.6 INNATE RESISTANCE

This is an age related insusceptibility of young ruminants of all domestic livestock, to infection with *Cowdria*, unrelated to the immune status of the dam. There is considerable resistance to disease in very young calves, lambs and kids (Uilenberg, 1981). However, different breeds seem to have their own level and age limit. For example, Neitz and Alexander (1941) found Freisland, Aberdeen Angus and Hereford calves up to the age of two to three weeks were resistant, while Uilenberg (1971), found that some calves succumbed to infection even below the age of three weeks. Creole goat kids up to 2 weeks (Camus, 1987) and Angora kids six weeks old

(Thomas and Mansvelt, 1957), were resistant, while merino lambs were resistant only up to nine days old (Uilenberg, 1971). Innate resistance wanes rapidly with age and Du Plessis and Malan (1987b), found that by one month susceptibility had increased by 22% in Freisland calves. The findings of Deem *et al.* (1996b), show that innate resistance is enhanced by the immunity of the dam. Henning (1956), and Uilenberg (1971), confirmed the resistance to heartwater disease of cattle raised in endemic areas. The lymphoid system of cattle and sheep has been found to be unique in containing a large number of γδ T cells (which form a high proportion of T cells in the neonate), which might play a part in the innate resistance of young ruminants (Hein and Mackay, 1991).

The resistance of cattle to heartwater is also thought to have non-specific components which takes effect in older animals (Soldan *et al.* 1993). These include substances like conglutinin (K), which was found to increase as the calves grew older, and whose level was shown to be correlated with the resistance of these calves to infection (Du Plessis and Bezuidenhout, 1979; Du Plessis, 1985).

Extrinsic factors affecting susceptibility of the host include the season and therapy with certain drugs e.g. Gloxazone. Dry season decreases food and increases stress and may cause vector concentration at watering points. Stressed animals are known to harbour more ticks. While the rainy season usually cause a general tick increase and build up, and hence also increase the level of challenge. Thus heartwater occurs through out the year in some zones but may decrease in incidence in the dry season while in some regions heartwater is seasonal and increases during the rainy season.

1.5.7 IMMUNITY

Observation in the field confirmed by laboratory experiments have shown that recovered animals develop immunity. There are aspects on the nature of immunity to heartwater that are still unclear. Studies on immunity to heartwater suggest that it is strongly dependent on cell mediated immunity. However there is concurrent an antibody response when most animals are immunized (Lawrence *et al.*, 1995a).

Immunity does not appear to be humoral for a number of reasons. Hyperimmune serum, and purified gamma globulins did not protect susceptible sheep against infection (Alexander, 1931; Du Plessis, 1970). Sera from immune animals did not neutralise elementary bodies of *Cowdria ruminantium* (Martinez *et al.*, 1993a, 1994). The gamma globulin response is not anamnestic and has been shown to drop in some cases after challenge of animals (Ilemobade, 1976; Du Plessis *et al.*, 1992b, Du Plessis and Malan, 1987d), and a considerable percentage of the animals that were serologically negative were also resistant to challenge (Du Plessis *et al.*, 1984).

However, antibody may have a role in the immune response as a degree of neutralisation was observed *in vitro* (Byrom *et al.*, 1993; Du Plessis, 1993), using serum of recovered animals.

There is mounting evidence on the role of cell mediated immunity in the protection against *Cowdria ruminantium,* such as the transfer of immunity to susceptible mice using Lyt-2^+ cells in mice (Du Plessis *et al.*, 1991, 1992). Cytokines, which have been shown to determine the type of immune response that ensues (Mosmann and Coffman, 1989), have been examined by a number of scientists for their roll in immunity to *Cowdria* (Totté, 1988; Totté *et al.*, 1990, 1993b, 1993c, 1994; Bensaid *et al.*, 1993; Mahan *et al.*, 1994a). Observations made from these studies have confirmed the importance of cytokines in T cell mediated immunity in cowdriosis and are guiding scientist in devising *Cowdria* vaccines based on killed elementary bodies and ultimately recombinant antigen vaccine.

The duration of immunity has been found to be variable (Neitz, 1939, 1968; Neitz *et al.*, 1947; Spruell, 1922; Henning, 1956; Haig, 1955; Stewart, 1987a; Du Plessis and Bezuidenhout, 1979), and to be accentuated by repeated exposure (Du Plessis *et al.*, 1992b). Although immunity was believed to be sterile for a long time, carrier state has been incontestably shown to exist in ruminants (Andrew and Norval, 1989; Camus, 1992), and mice (Du Plessis, 1982; Wassink *et al.*, 1990). Artificial sterilisation of immune sheep (Du Plessis, 1981a), was thought to be the cause of their increased susceptibility to homologous challenge. Premunition may explain the observation of apparent relapses in recovered animals by some scientists (Alexander, 1931; Neitz and

Alexander, 1945; Haig, 1955).

1.5.7.1 IMMUNOGENIC AND ANTIGENIC VARIATION

Antigenic differences between stock were known to exist but immunogenic differences were thought not to be important (Van Winklehoff and Uilenberg, 1981, Uilenberg *et al.*, 1983). This led to the belief that the Ball 3 strain could immunise and protect against all other strains of *Cowdria*. Recent studies have shown that there is great immunological diversity between strains (Du Plessis *et al.*, 1989; Jongejan *et al.*, 1988, 1991b; Stewart, 1989; Brown *et al.*, 1989), even with *Cowdria* isolates obtained from one farm (Du Plessis *et al.*, 1992b). They have also indicated that the most virulent stocks produced protection against a broader spectrum of other isolates (Du Plessis *et al.*, 1989; Jongejan *et al.*, 1991b). These immunologic differences are thought to be the reason why heartwater occurred in immunised animals in the field (Du Plessis, and Van Gas, 1989; Uilenberg and Camus, 1993).

1.6 Disease control and prevention

Disease control is approached in four basic ways: vector control, immunisation, chemotherapy and use of natural resistance (of young animals and indigenous breeds) (Uilenberg, 1990). The success of disease control is greatly influence by the attitude of farmers to the different methods (Spickett and Fivaz, 1992a & b).

1.6.1 VECTOR CONTROL

Conventional tick control is by regular acaricidal treatment of domestic livestock against tick infestations which must be intensive, if tick transmission of cowdriosis is to be prevented. This method of disease control is fraught with many difficulties and does not provide a long term solution (Walker, 1984; Norval *et al.*, 1991). Acaricide resistance in ticks continues, cost of acaricides and tick control installation is high and the acaricides are environmental pollutants. New pour-on acaricides may waive some of these difficulties, but do not diminish acaricide resistance. When effectively carried out, it leaves the animals highly susceptible to all tick borne diseases while upsetting endemic stability (for both heartwater and other tick borne diseases) (Norval *et al.*, 1991). Strategic acaricide application may be the more rational approach for

controlling tick infestations and of maintaining endemic stability (Bezuidenhout and Bigalke, 1987). However, *Amblyomma variegatum* tick eradication is recommended in the Caribbean islands (Drummond and Butcher, 1988), because of the isolated nature of the islands, dermatophilosis which is associated with the *Amblyomma* tick and threat heartwater poses to the American mainland (Barré *et al.*, 1987).

Pasture management and breeding of cattle for tick resistance (Walker, 1984; Young *et al.*, 1988), may also reduce the level of tick infestation but it does not prevent transmission.

1.6.2 IMMUNISATION

Immunisation in heartwater has until recently, been most practical and effective only by infecting an animal with infected blood and subsequently treating it after the onset of disease. Since elementary bodies became available in culture, an attenuated EBs vaccine not requiring treatment has been used, and inactivated EBs have yielded promising results.

1.6.2.1 BLOOD VACCINE

The 'infection and treatment' immunisation was first developed by Neitz and Alexander (1941, 1945), and remains the most used method for immunising livestock to date. The procedure has not changed up to today: it is cumbersome and risky (Uilenberg, 1981). Animals may fail to become protected against homologous challenge and field challenge (Neitz and Alexander, 1945; Gueye *et al.*, 1989). One of the causes no doubt, is due to the occurrence of immunovariants (Jongejan *et al.*, 1988, 1991b, 1993b; Du Plessis *et al.*, 1989). Attempts to improve on the vaccine by combining a number of stocks, (Du Plessis *et al.*, 1990b; Jongejan *et al.*, 1991b) were unsuccessful. Oberem and Bezuidenhout (1987b), standardised the method of blood vaccine production and use, which has recently been reviewed by Camus *et al.*, (1996).

1.6.2.1.1 THE VACCINE AND PROCEDURES

The Ball 3 vaccine is the only blood vaccine which is widely used in the Southern African countries and South Africa. Bezuidenhout *et al.*, (1987), investigating the route of infection, found the i/v route most reliable and the i/m route promising when certain additives were included in the inoculum. The site of the i/v, route may not change the outcome as demonstrated by Arnold and Asselbergs (1981), when they used the ear vein.

Vaccine produced from infected sheep blood (to avoid including other haemoparasites for cattle), requires a cold chain for its preservation and use. Different batches may give slightly different reactions in groups of animals (Lawrence *et al.*, 1995b). Modifications have been devised for field immunisation of large numbers of animals to improve handleability and efficiency of immunisation. Treatment does not interfere with the establishment of immunity (Simpson and Wiley, 1951), unless given too early. Animals are treated with long acting tetracyclines after a period determined by individual animal monitoring (Arnold and Asselbergs, 1981; Van der Merwe, 1979, 1987) or by predetermining the day of treatment for the whole group (Neitz and Alexander, 1945, Fick and Schuss, 1952, Poole, 1962a & b; Du Plessis and Malan, 1987), or determining the day of treatment by monitoring a small but representative group of the immunised animals (FAO, 1984): the efficacy and efficiency are affected by these compromises (Lawrence *et al.*, 1995b). A degree of ineffectiveness of the i/v inoculation seen particularly in cattle (Alexander, 1931; Haig, 1955; Uilenberg, 1971), has prompted the use of a repeat immunising inoculum (Van der Merwe, 1979), and been found to improve the immunisation efficiency.

Heartwater is a dose dependent disease but the effects of dose is most readily seen when EBs from tissue culture, which achieves a highly infective dose in a small volume is used, leading to an incubation period as short as 5 days. Albeit for field use a volume of 5 mls of the Ball 3 vaccine in sheep and 5 to 10 ml in cattle has been recommended (FAO, 1984), 2 ml of inoculum has initiate an infection successfully (Arnold and Asselbergs; Camus *et al.*, 1996). The effects of dose are less marked when infective blood is used and small volumes of blood have yielded similar

infectivity as large volumes.

1.6.2.2 GUTS VACCINE

A tick derived vaccine was developed by Bezuidenhout (1981), and offers many advantage over the blood vaccine including ease of production, cost and storage (Camus *et al.*, 1996). It was found to yield equally good results (Bezuidenhout and Spickett, 1985, Van Der Merwe, 1987), as the blood vaccine. But it still requires intravenous inoculation and intensive monitoring. It was refreined from being used because it caused shock to some young lambs and kids (Van Der Merwe, 1987).

1.6.2.3 TISSUE CULTURE ATTENUATED VACCINE

One of the fruits of successful *Cowdria* culture (Bezuidenhout *et al.*, 1985), was the development of an attenuated tissue culture vaccine (Jongejan, 1991). Its main advantages are a lack of necessity for a therapeutic drug and it provides solid immunity against virulent homologous challenge (Jongejan, 1991b, 1993b).

1.6.2.4 INACTIVATED VACCINE

The use of inactivated EBs is a step towards the use of a recombinant vaccines (Martinez *et al.*, 1993, 1994; Mahan *et al.*, 1995). Promising results have been obtained but the necessity to incorporate Freunds adjuvant which has adverse side effects (Mahan *et al.*, 1995), makes the use of inactivated EBs impractical until a suitable adjuvant is found. An interleukin IL-12 has been found to have good adjuvant potential with accellular antigens (Mahon *et al.* 1996). The use of the 31 kDa antigen did not resulted in protection against heartwater (Van Kleef *et al.*, 1993).

1.6.3 CHEMOTHERAPY

A sulphonamide drug, Uleron offered the first effective anti-*Cowdria* therapy (Neitz, 1939). Other sulphonamides were subsequently shown to have therapeutic effects (Alexander *et al.*, 1946; Neitz, 1968), but it was tetracycline that achieved the most satisfactory results (Weiss *et al.*, 1952; Haig *et al.*, 1954; Uilenberg, 1971). Long acting tetracyclines are particularly useful because they abrogate the necessity for repeated treatments (Gueye and Vassiliades, 1985). An anti-*Cowdria* drug called

rifampicin was discovered but has not yet been registered for field use, (Oberem and Mathee, 1988). Gloxazone was effective in sterilising infection (Du Plessis, 1981; Stewart, 1987), but has been found too toxic to be released for use. The mouse model has been used to screen for drugs against heartwater (McHardy and MacKenzie, 1984, 1987).

1.6.3.1 CHEMOPROPHYLAXIS

Under field conditions, heartwater progresses too rapidly for treatment to be successful (Uilenberg *et al.*, 1983). This may explain why Cassard (1957), was unable to save any of twelve Friesians that had acquired heartwater naturally. The use of drugs is thus relegated to experimental cases and in prophylaxis (as in immunisation), or in the face of an outbreak of heartwater (those animals that are still incubating the disease).

In pure prophylaxis, susceptible animals are protected by sustained drug cover (e.g. with a long-acting oxytetracycline), for a duration of 4 weeks, while limiting disease exposure from ticks (Purnell, and Schroder, 1984; Purnell, 1984). The 'blanket cover' is expensive but has met with some success. However it requires areas of heavy tick challenge to ensure that animals become infected during the 4 week period.

1.6.4 RESISTANT ANIMALS

The natural but temporary resistance of young animals and resistance of indigenous breeds (Uilenberg, 1990), has been exploited when immunising animals against heartwater. Alexander and Neitz (1941), made use of this fact in their initial immunisations of calves.

The observation that there are breeds with high resistance to *Cowdria* in nature has prompted scientists to think about natural selection in the resistance of animals (Matheron *et al.*, 1987, 1991) and to simulate and enhance selection of resistant animals artificially with good prospect for success in Creole goats (Matheron *et al.*, 1987, 1991). However attempts to breed for resistance to *Cowdria* in mice was not fruitful (Du Plessis, 1983; Du Plessis *et al.*, 1990a) perhaps because laboratory mice are highly inbred and already posses highly selected traits.

1.7 Conclusions

Research on heartwater has produced a lot of new findings about *Cowdria* and the disease it causes (Bezuidenhout, 1993; Camus *et al.*, 1996). Serological tests have been developed albeit there is still a problem of specificity. Molecular characterisation has advanced our knowledge on characteristics of important proteins of *Cowdria* at the genetic level and revealed a close relationship of *Cowdria* particularly with the *Ehrlichia*. However, information is still needed on a number of areas. One is on immunity: the reason(s) why one stock may protect against another while the other stock protects against it partially or not at all. In what ways should scientists manipulate the immune system with inactivated (and ultimately bio-engineered antigen) vaccine to produce not just immunity but cross protection against virulent agents that may be in the field. Vertical transmission in heartwater epidemiology and immunity should be further investigated.

Under the conditions prevailing in Africa, a recombinant vaccine may not be available or appropriate to solve the current and potential threat of heartwater. Steps need to be taken urgently to avert potentially explosive situations using more readily available techniques. Any one of the control methods may not be adequate to control heartwater on its own (other tick borne diseases need to be taken into consideration also), but an economic and robust integrated control is likely to result when several of the methods are combined (Young, *et al.*, 1988; Anon, 1989).

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 *Cowdria*

Cowdria was isolated from 8 localities in 8 districts as reported below. Two isolates each were obtained from Tana River and Machakos districts. The total number of Kenyan isolates used in this study were eleven viz.:- Bamba, Galana 229, Galana 233, Kathiani 32, Kathiani 972, Suswa, Isiolo, Marigat, Baragoi, and Asembo were all newly made isolates. The eleventh isolate, the Kiswani stock (Kocan *et al.*, 1987b), was provided by Dr Morzaria of ILRI. The first passage was used unless there was an insufficient amount for various reasons and then the second passage was used. (details of stabilate preparations and storage can be found in Section 3.2.6).

2.2 Experimental sheep

Heartwater susceptible, pure Corriedale wethers or female cull sheep, 5 to 12 months of age were purchased from two farms located at high altitudes less suitable for the survival of *Amblyomma* ticks (Walker, 1974), and practising good tick control and with no recent history of heartwater. Animals were examined thoroughly for *Amblyomma* before purchase and tested for presence of antibodies to *Cowdria* by the cELISA test using the El5 *Cowdria* antigen to ascertain they had no previous exposure to heartwater. On arrival at the station, they were clinically examined and rectal temperature taken. They were tagged, dewormed with a broad spectrum anthelmintic, Levamisole HCl. (Nilzan ® ICI, England), visible ticks removed (for later speciation), then rubbed with acaricide Deltamethrine Pour on (Spot on®), or sprayed with Steladon®. They were vaccinated against clostridial diseases such as black leg, enterotoxaemia and gas gangrene.

They were housed in tick proof pens, individually or in groups of 10-15, at the National Veterinary Research Centre, Muguga, Kenya Agricultural Research Institute. They were acclimatised for a minimum of one week and fed on grass and Lucerne hay, a ration of sheep pellets and a commercial mineral salt lick ((Maclik®, Cooper Kenya Ltd.). Water was provided *ad lib.*

Twenty nine sheep were used for isolation and isolation attempts. Sixty one sheep were used in primary infection with blood stabilates. (These included sheep used for

making first passage stabilates, first time stabilate testing, positive controls and tick infectivity testing). Eighty sheep were recruited in the two way cross immunity trial. Thirteen sheep were replaced during immunisation for those that died.

2.2.1 RANDOMISATION OF SHEEP

The block randomisation method (Bancroft, 1968), was used in large experimental groups. Briefly, the sheep were ear tagged and weighed. They were then arranged by order from the heaviest to the lightest and assigned random numbers from a scientific calculator (Casio fx - 82 LB Fraction). The random numbers were used to obtain two controls (for homlogous boost) for each of the two immunisation groups by sorting and selecting the four smallest random numbers. Every alternate random number was put into one group the first pair going to the first immunisation group and the other pair going to the second immunisation group. The remaining sheep were rearranged according to weight and blocked in twos. The two immunisation groups for each half of the immunisation trial were obtained by assigning the sheep in each paired block into two groups: all the sheep with the smaller random numbers were placed into one group and the larger random numbers were placed into the second group. The 4 challenge groups within each immunisation group were obtained by arranging the sheep of that immunisation group in order of descending mass and blocked in fours. Using the same random numbers, the smallest number was assigned to group one the next to group two and so on until there was four challenge groups of seven sheep each. The two smallest random numbers in each challenge group were used to select the sheep to act as challenge controls for the challenge group. (Deselection of some control sheep was also done by using the random numbers)

2.2.2 INFECTION OF SHEEP WITH *COWDRIA*

After isolation, infection of sheep with *Cowdria* was carried out using blood stabilate made on the second day of high fever ($\geq$ 41°C) in the isolation animals and stored. Each sheep was clean shaven on the neck area for easy access of the jugular vein. The stabilate which is stored in 3.6 ml or 4.5 ml cryotubes tubes was quickly thawed from liquid N_2 by emersing the tube into a water bath at 37°C and shaking from time to time. The stabilate was decanted into a bijou bottle kept cool on crushed ice. The

sheep was restrained and 1 millilitre of stabilate inoculated into the jugular vein of the sheep with minimum delay aspirating at the beginning and at the end of inoculation to ascertain it had been injected intravenously. The sheep were observed for a few minutes for possible anaphylactic reaction and treated with Atropine sulphate when this occurred.

2.2.3 MONITORING OF EXPERIMENTAL SHEEP

All sheep were monitored by taking rectal temperature daily early in the morning and observing animals individually for clinical signs using a check list (Appendices 4.1a & b). Sheep were bled clotted blood for serum before infection, and day 28 post infection, and on other days as indicated in individual protocols. Unclotted EDTA or Lithium Heparin blood was collected for culture work as indicated else where, and for haematology twice a week when this was needed.

2.2.3.1 ESTIMATION OF THE REACTION INDEX

A reaction index (RI), was estimated according to Du Plessis (1985). The reaction index took into consideration the febrile reaction and the eventual death or recovery of a sheep. The contribution of the febrile reaction to the reaction index was calculated by determining the mean daily temperature from day one of infection to onset of the febrile reaction. The sum of the recorded temperature increases in °C, above the average, prefebrile temperature, was recorded for after start of the reaction, and estimated to give the reaction index due to fever. The contribution of the eventual death or recovery to the reaction index was calculated by awarding no points to an animal that did not die, and awarding thirty six points to sheep that died on day six after the start of febrile reaction. The thirty six points were increased by three points for every day less than six days that the animal survived, and reduced by three points for every day more than six days that the animal had survived. The reaction index was the total sum of the febrile reaction and fatality (duration of survival), index. The magnitude of the score an animal could earn on the febrile reaction was increased by a low mean pre-febrile temperature, and the magnitude and persistence of the fever. A sheep that did not reach the cut off level (40°C), was not awarded any points towards the febrile response. The maximum a sheep could earn on the fatality score is if it died

suddenly without fever being detected and so obtain a score of 18 points (6x3), to add to the 36 basic points awarded for fatality (= 54). The least a sheep could earn on the fatality score would be if it died 18 days after the start of fever (12 days after the reference 6 days), and therefore earn a score of zero. Six days was taken as the reference point because it was the median for the fever (Tables 4.2a & b combined) and each of the sheep had reactions well within the scope of this scoring system without earning negative points.

2.2.4 EXTRACTION OF *COWDRIA* DNA FROM SHEEP BLOOD

Cowdria DNA was extracted from whole fresh blood by adding 500µl, 0.83% NH4Cl to 500µl of blood to lyse and remove red cells. The lysate was centrifuged at 13,000g for 2 minutes and the pellet washed twice in PBS. 100µl TE buffer containing 100 µgml-1 Proteinase K was added and the samples were digested at 55 C for 1 hour. The protease was inactivated by heating at 95 C for 10 minutes and the sample was then aliquoted until used for PCR. Mature neutrophil cultures were washed twice, then treated the same way as the blood extract. Purification of *Cowdria* DNA by phenol/chloroform extraction was found to add no advantage for blood extracts.

2.3 Experimental mice

Balb/C strain of the albino white mice, 8-12 weeks of age were obtained from the ILRI colony. They were examined for health and put in fives or sixes per cage. They were each given a number identified with a cage number by using colour dye to mark standard body parts. They were maintained on mice pellets (Unga Co. Kenya), and water, and wood shaving bedding changed 3 times a week. Mice were euthanised after an experiment by cervical dislocation. Mice were bled pre-infection and post-infection for serum by a tail snip and gentle stroking into a capillary tube.

2.3.1 INFECTION OF MICE WITH *COWDRIA*

A mouse was restrained in a modified 50 ml syringe to access the tail and minimise mouse movements while being injected. The tail was warmed in a 37°C water bath for a few minutes to dilate the veins and swabbed once or twice with methylated spirit in cotton wool. A one millilitre syringe and gauge 25-27 needle was used to aspirate 0.2

ml of blood or stabilate from a container and inoculated into a tail vein. The mouse was marked on the back with a second dye to indicate successful intravenous inoculation or left without if otherwise. The mouse was returned to its cage and monitored daily.

2.3.2 MONITORING OF MICE INFECTED WITH *COWDRIA*

Mice were observed for clinical manifestations such as anorexia, piloerection, motion and changes in behaviour every day.

2.4 Post mortem examination

Sheep that died or were put down on humane grounds were examined for gross lesions resulting from cowdriosis. Brain crush smears were made to make a confirmatory diagnosis of *Cowdria ruminantium* by examining Giemsa stained smears under the light microscope (Leitz, Laborlux, Germany), first under x10 magnification, then under x50 or x100 magnification under oil emersion.

Mice that died were also post mortemed and observed for gross pathology of internal organs, such as presence of hydrothorax, ascites, splenomegally and hepatomegally as well as presence of rickettsial organisms (colonies), at the microscopic level in brain squash smears, and impression smears of the liver lung, spleen and kidneys. They were stained with Giemsa and examined for presence of *Cowdria* colonies under the light microscope. Hydrothorax was taken to be pathognomonic in mice (Prozesky and Du Plessis, 1985a).

2.5 Serology

ANTIGEN

A soluble *Cowdria ruminantium* (Welgevonden), antigen called EL5 was prepared and supplied by Dr Keith Sumption of the CTVM. Briefly, TC EBs were harvested and washed twice in PBS. The EBs were resuspended in lysis buffer (Appendix 2.2) incubated at 37°C. The supernatant was used as antigen to coat ELISA plates.

SERUM

Blood of ruminants was collected in the field and from experimental animals before infection and at various intervals after infection, via the jugular vein using a sterile vacutainer needle and tube. The blood was allowed to clot at room temperature then centrifuged at 2,700g for 15 minutes (Chilspin, MSE). It was aliquoted into 1 ml serum storage tubes and assigned a serum number then frozen at -20°C until needed. The sera were tested in the cELISA test as developed at the CTVM (see below).

The blood of mice was collected before the mice were infected and on day 28 post inoculation or post challenge. The mice were restrained in a modified 50 ml syringe and the tail was swabbed with spirit and the tip snipped slightly. The blood was massaged from the tail by strokes and collected into a capillary tube (containing Lithium heparin), drop wise until it was two thirds full. The capillary tube was marked to indicate the mouse and cage number it had come from. After sealing with Crystaseal® sealant, the capillary tubes were spun in a micro-haematocrit centrifuge (Hawksley, England), and plasma harvested by breaking the capillary tube at the buffy coat plasma interface and blowing it out into a microtube (Alpha laboratories, UK), for storage. This provided about 20 µl serum/plasma. Sample details were recorded on the tube as well as the tube rack card and record sheet.

2.5.1 THE COMPETITIVE ENZYME LINKED IMMUNOSORBENT ASSAY (cELISA) TEST

Serum bled from experimental sheep and also that from cattle and goats in the field were tested against the EL5 (soluble *C. ruminantium* antigen). Other reagents were competitor made from a selected whole immune serum, anti-species conjugate and a chromogen TMB (Tetramethyl benzidine)(Sigma).

2.5.1.1 THE cELISA TEST FOR HEARTWATER

A 0.05M solution of Carbonate buffer pH 9.6 at 25°C was prepared and used for diluting the EL5 antigen at 1/3000 for the number of plates to be tested.

Special 96 well ELISA plates (Dynatech, Immulon), were filled with 100µl dilute antigen per well using a multi-channel pipette (Titretek plus). One, no antigen control

well was left without antigen and the plate sealed with Avery P.55 plate sealer (Flow Laboratories), and incubated flat overnight at 4°C.

The seals were removed and excess antigen washed off with sterile half strength Phosphate buffered Saline with 0.05% Tween 20 (PBST 0.05%), pH 7.3 four times and dried by banging the plates onto paper towels.

Fifty microlitres of test serum or positive or negative control sera was added to the wells according to the test format. Fifty microlitres PBS was added to the no serum control wells then fifty microlitres of biotinilated competitor serum diluted 1:25 with PBS 0.1% Tween 20 added to all wells. The ELISA plates were incubated for 1 hour at 37°C on the shaker incubator (Varishaker, Dynatech). Plates were again washed four times as before and dried on blotting paper. A 100 µl of 1:2,500 dilution ExtrAvidin-peroxidase conjugate in PBS 0.1% Tween 20 was added and plates incubated on the shaker incubator for 45 minutes. The plates were again washed as before. One tablet of Tetramethyl benzidine (Sigma code T3405), chromogen (TMB), was dissolved in phosphate citrate buffer and 1 µl hydrogen peroxide substrate per millilitre of solution added mixing the two thoroughly immediately before use. 100 µl was added per well and colour allowed to develop for 8 minutes. The reaction was stopped using 100 µl of 1M sulphuric acid. The plate was blanked on the no antigen well and read on an ELISA reader (Titertek Multiscan® MCC/340), using a 450 nm filter. The readings were converted to percent inhibition and a 70% inhibition cut off used to interpret the readings to determine the positive ($\geq$ 70%), and the negative sera ($<$ 70% inhibition).

$$\% \text{ inhibition} = \frac{\text{Mean OD PBS control - OD sample}}{\text{Mean OD PBS control}} \times 100$$

A condition for accepting the readings was that the PBS wells (no competition), should be 0.9 or higher, and the two PBS wells be within 10% of each other . The positive control well had to be $<$ 0.1.

2.5.2 THE INDIRECT ENZYME LINKED IMMUNOSORBENT ASSAY (iELISA) TEST

Mouse sera were tested in the iELISA test. The soluble EL5 *Cowdria ruminantium* (Welgevonden), antigen prepared at CTVM as above, anti-mouse Horse radish

peroxidase (HRP), conjugate at 1:1000 dilution and a chromogen TMB were used.

The iELISA cut off level was determined for an uninfected (normal), mouse population as follows. Optical density values (OD), from one hundred and fifty sera collected pre-infection from the Balb/c mice used in various experiments, was used to obtain a 95% cut off OD level to determine the values that were positive or negative among the sera collected from the mice post infection (> 28 days pi). Sera with OD values above this level were considered to be positive i.e. due to a serological response, while those below it were considered to be negative. This value was found to be an OD value of 0.143. A pool of serum from ten uninfected mice was found to have an OD of 0.037 in the same test.

2.5.2.1 THE I-ELISA TEST FOR ANTIBODIES TO *COWDRIA RUMINANTIUM*

An ELISA plate (Dynatech, Immulon), was coated with EL5 antigen all wells being coated at a dilution of 1:6000 overnight at 4 C. The excess antigen was washed off four times and dried as in the cELISA.

Sera (first antibody)were diluted at 1:100 dilution and added to the plate in duplicate. Positive and negative control sera were also diluted at 1:100 and put into control positions. The plates were covered and incubated for 1 hr at 37 C in a Varishaker Incubator (Dynatech). The plates were washed and dried as in the previous step. Anti-mouse IgG HRP (second antibody), diluted at 1:1000 was added to all wells and incubated as before in the shaker incubator for 1 hour. The washing was repeated. The substrate (H_2O_2), and chromogen (TMB), were then prepared and 100 µl of the mixture added. Colour was allowed to develop for 6 minutes then stopped using 1M sulphuric acid. An ELISA reader (Titertek Multiscan® MCC/340), blanking on the PBS well(s), using a filter of wavelength 450 nm was used to read the plate and the results printed off a 24 pin printer (OKI Microline 390 Elite). All the results were corrected by a factor on the reader (= mean of positive control of reference plate/ mean of positive controls of plate being read), so that all plates were compared at the same level.

The average of the duplicate readings was calculated for each serum and recorded on an ELISA sheet. The cut off value (see above), was used to determined which serum

was positive and which was negative.

2.6 Western blotting

Solublised proteins of endothelial *Cowdria* culture (Welgevonden stock), prepared at the CTVM were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), 12% gel using a MiniProtean® II system and subsequent Western blotting. Electrophoretic transfer was carried out at 200 volts for 45-60 minutes using a semi-dry cell (Trans-Blot® Bio-Rad). The blots were quenched in 5% skimmed milk in blocking buffer for 2 hours and incubated with test serum (sera from sheep recovered from different Kenyan isolates), positive or negative control serum diluted at 1:100 in 5% skimmed milk (Marvel®), in blocking buffer for twelve hours (overnight). Bound antibodies were visualised by incubating with rabbit anti sheep (1:2000), peroxidase (HRP) (Sigma®), diluted in blocking buffer containing 5% skimmed milk for two hours. After washing, bound conjugate was visualised by addition of α chloro-napthol dissolved in cold methanol and diluted in PBS and supplemented with H_2O_2. The reaction was stopped after full colour development in 5-20 minutes by thorough washing with milliQ water. The blot was photographed as soon as possible after development with a strip of paper next to the blot on which molecular weight markers were marked.

2.7 Culture of *Cowdria*

2.7.1 NEUTROPHIL CULTURES OF *COWDRIA*

2.7.1.1 PREPARATION OF NEUTROPHILS

Blood obtained by venapuncture of the jugular vein into 2 sterile 5 ml or 10 ml EDTA vacutainer tubes (Vacutainer systems -New Jersey), was processed using a modification of the method desribed by Carlson and Kaneko (1973). Briefly, it was spun at 1,000 x g (23,000 rpm), for 20 minutes in a cooled centrifuge (IEC Centra-7R refrigerated centrifuge USA), and plasma, buffy coat, and top quarter of red cells discarded. The remaining packed red cells were diluted with 2 ml PBS then lysed by hypotonic lysis by mixing with 12 ml sterile distilled H_2O. After 30 seconds of gentle mixing, 6 ml 2.7% sterile NaCl solution was added to restore isotonicity. The lysate

was spun at 200 x g for 10 minutes and neutrophils washed twice in 20 ml of sterile PBS. To do this the supernatant was tipped off and the pellet re-suspended in PBS and centrifuged at 200 x g for 10 minutes each time.

2.7.1.2 GROWTH OF *COWDRIA* IN NEUTROPHIL CULTURES

The neutrophils were re-suspended at 1-2x 10^6 / ml in 5 ml RPMI 1640 complete medium containing 10 % Fetal Calf Serum (Myoclone GIBCO BRL), 200 mM L-glutamine, 100 IU/ml penicillin, 25 µg/ ml streptomycin 25 µg/ml fungizone and incubated in a humidified, 5% CO^2 incubator. Two thin cytospin smear were made immediately, and every 24 hrs from the day of preparation up to the third or 4th day of culture for each culture. Briefly, two drops from cell suspension were spun at 1,000 rpm using a cytocentrifuge (Cytospin, Shandon and Southern). The cells spread on the cytospin smears were air dried, fixed with methanol and stained with Giemsa. They were examined for morulae formation under oil emersion at x100 magnification using a light microscope (Leizt, Leibovitz, Germany). The culture was terminated when the neutrophils were considered too deteriorated, which was usually on the 3rd or 4th day.

2.7.2 ENDOTHELIAL CULTURES OF *COWDRIA*

A number of methods were employed in the endothelial culture attempts. Ten millilitres of blood was collected, from pyrexic sheep infected with *Cowdria,* into vacutainer tubes containing Lithium heparin. In the first method the blood was passed through a syringe and needle a couple of times and 2 ml of the lysate inoculated onto a confluent endothelial monolayer in a 25 cm^2 Sterilin® flasks. This was incubated at 37 C on a rocking platform (24 cycles /hr), for 2 hours. The blood inoculum was washed off with PBS three times and the culture incubated with fresh culture medium.

In the second method, the blood was left overnight and washed off the following day. In the third, infected neutrophil cultures were used as inoculum instead of the blood.

And in a fourth, 2 ml plasma separated from fresh blood was used as the inoculum. The inoculum was poured off the following day without washing and replaced with 5 ml GMEM medium supplemented with Myoclone fetal calf serum, glutamine and

penicillin. Fungistat was used only when it was thought to be necessary. The culture was incubated in a 5% CO_2 incubator. Every 3-4 days 2.5 ml of medium was removed and replenished with fresh medium. Alternatively, L-15 medium was used to replace the inoculum after seeding. The L-15 cultures were not put in a CO_2 incubator but were kept on a rocking platform when culturing for *Cowdria*.

2.8 Amplification of *Cowdria* DNA by polymerase chain reaction

2.8.1 PRIMER DESIGN

Two sets of primers were used in these studies (Table 2.1):

The first set of primers were based on the pCS20 DNA probe sequence (Waghela *et al.*, 1991; Mahan *et al.*, 1992). These were obtained from one of two open reading frames (ORF), of a 1,306 bp clone of *C ruminantium* (Crystal Springs), DNA sequence called pCS20 (Waghela *et al.*, 1991). The AB128/AB129 primers amplify a 279 bp product. The second set of primers, the HE primers based on the 16S rDNA primers (adopt their nomenclature from Anderson *et al.* (1992)), were derived from the 16S rRNA gene sequence of *Cowdria ruminantium* (Crystal Spring) (Dame *et al.*, 1992). These are HE1(cr), HE2, HE3(s), HE3(l), primers (Watson, 1993), and a modified HE1 primer, HE1(cow), which is predicted to amplify 352 bp - 388 bp products. The 16S rRNA gene sequence of the Crystal Spring stock of *Cowdria ruminantium* (accession number X61659), was aligned with the 16S rRNA gene sequence of that of *Cowdria ruminantium* (Omatjenne agent) (accession number U03776), to deduce the HE1(cow), sequence. *C ruminantium* Senegal (accession number x62432), and Crystal Spring stocks had identical sequence for the new primer.

HE1(cow), was designed by adding six extra bases from the *C. ruminantium* (Crystal springs), gene sequence to the 3′ end of HE1(cr), to increase match (and add stability in PCR reactions), with the *C. ruminantium* (Omatjenne), gene sequence. The pair of HE1(cr), and HE3(s), amplify a 388 bp product, and the product of HE1(cow/ HE3(s), and HE1(cow)/HE3(l) are very similar to that above. The HE2 and HE3(l) set amplify a 352 bp product, because HE2 is located to the 3′ end of the HE2 site.

A pair of universal primers known to amplify > 90% of the, 16S rRNA gene length (of most if not all eubacteria, Wilson *et al.*, (1990)), adopted by Anderson *et al.*, (1992), were used to set up nested PCR. These were the EC11 and EC12 which amplify a 767 bp fragment.

Tick DNA was amplified using newly formulated primers from an *Amblyomma variegatum* gene sequence as a pair amplifying a 240 bp product. They were designated Avar-F1 (forward primer) and Avar-R2 (reverse primer) (Table 2.1).

2.8.2 SETTING UP A PCR REACTION

A master mix of the reaction constituents was made such that each 50µl PCR reaction contained 10 mM Tris HCl, pH 8.3: 50 mM KCl, 3.0/4.0 mM $MgCl_2$, 0.001% w/v gelatine, 200µM of each dNTPs, 0.5µM each of primers AB 128 and AB129, or 0.2µM of HE1(cow) and HE3(s), or Avar-F1 and Avar-R2 primers, 1.25 Units of UltroTAQ DNA Polymerase (UltroTaq™ 5U/µl, Bioprobe or ThermoMetric, UK). The PCR master mix was prepared for the required number of samples plus an extra one in every ten to cater for inaccuracies which could occur during pipetting. Volume adjustments were made to accommodate different reagent strengths for different PCR requirements. The Taq polymerase was added last, just before aliquoting the master mix to the samples and all reagents and stocks were kept on ice until ready to amplify.

The sample was either introduced under the mineral oil and denatured in that environment from the start of the PCR or denatured first (tick derived DNA), before the master mix and mineral oil were added. Viz.: Five microlitres of sample was transferred to labelled tubes and denatured at 96°C (constant), for 10 minutes on the thermal cycler (Omnigene™ Thermocycler amplifier Hybaid). The samples were cooled on ice for 10 minutes and pulsed down on a micro-centrifuge, followed by the addition of 45µl of master mix and then each was over-layed with 50 µl (two drops), mineral oil (molecular biology grade, Sigma, UK). The reactions were performed for 30-45 cycles programmed in a thermocycler (Omnigene™ amplifier, Hybaid), comprising three steps at temperatures selected to be suitable for amplification conditions required for a set of primers.

2.8.3 DETECTION OF A PCR PRODUCT

A 10 x 15 cm gel casting tray was strapped with autoclave tape on both ends and set on a levelling platform. 1.96g or 2.56g of type 1 agarose powder (Sigma, UK), was weighed and dissolved in 130 ml x1 TBE buffer (Appendix 2.), in a microwave oven (or hot plate), to make a 1.5%-2% agarose. It was allowed to cool slightly to about 70°C and 6.5µl of a 10 mg/ml ethidium bromide solution (0.5µg/ml final concentration), was added and the gel cast to set with a 20-30 gel comb in place. The gel was placed in an electrophoresis apparatus and the tank filled with 1x TBE buffer. Twenty microlitres of PCR product was transferred into a 0.6 ml Eppendorf tube containing 2 µl x6 DNA loading buffer (Appendix 2.), and fifteen to twenty microlitres of this mix loaded into a well each until all samples had been loaded. One or two wells were loaded with molecular weight marker (1,2,3 Kb Ladder, Appendix 2.), to determine the size of the products produced. The gel was electrophoresed at 80V (and 0.9 amps), for 45- 60 minutes or until the blue front indicated a satisfactory distance had been travelled by the products to allow good resolution. The gel was then photographed with a Polaroid camera over a UV illuminator and red filter to obtain a permanent record and to estimate the size of the PCR product.

2.8.4 PRECAUTIONS TO MINIMISE CONTAMINATION

Reagents were aliquoted and made up in new sterilised tubes using new sterilised tips and pipettes subjected to UV sterilisation (GS Genelinker™, Biorad), in a room where work on *Cowdria* had never been performed. Gloves were worn at all times. Glassware, instruments and reagents were not moved between different rooms used to perform this work. Pipettors, tube racks, ice buckets were dedicated to one job.

Samples were added in another room not routinely used to process or deal with the heartwater agent. The PCR products were handled in yet another room and clothing used in this room was not worn in the other rooms to avoid contamination with PCR products. Used tips and tubes were discarded and autoclaved/incinerated.

Table 2.1: DNA primers their sequence and theoretical amplification specificity

Primer code	Sequence	Theoretical primer specificity
	pCS20 derived primer set from 5′-3′	
AB 128	ACTAGTAGAAATTGCACAATCTAT	*C ruminantium* (Crystal Spring) (Mahan *et al.*, 1992)
AB 129	TGATAACTTGGTGCGGGAAATCCTT	*C ruminantium* (Crystal Spring)
forward	HE primers derived from the 16S rDNA sequences from 5′-3′	
HE1(cr)	CAGTTATTTATAGCTTCGGCTATGAG	*C ruminantium* (Crystal Spring) (Dame *et al.*, 1992)
HE1(cow)	CAGTTATTTATAGCTTCGGCTATRAGTATCTG	*C ruminantium* (Senegal, Crystal Spring)
HE2	GTGGCAGACGGGTGAGTAATGC	*E. canis, E. phagocytophila, E. chaffeensis, E equi, E. ewingi, E. risticii, C ruminantium* and *Anaplasma marginale*
reverse		
HE3(s)	GGTACCGTCATTATCTTCCC	*E. canis, E. phagocytophila, E. chaffeensis, E equi, E. ewingi, E. risticii, C ruminantium* and *Anaplasma marginale*
HE3(l)	CTATAGGTACCGTCATTATCTTCCC	*C ruminantium* (Senegal, Crystal Spring), *E. canis, E. chaffeensis, E. ewingii*
	EC primers derived from the 16S rRNA gene	
EC11	AAGGATCCGGACTACHAGGGTATCTAAT	universal bacteria (Anderson *et al.*, 1991)
EC12	AATCTAGAGTTTGATCMTGG	universal bacteria (Anderson *et al.*, 1991)
	Avar primers derived from the 16S rRNA gene (of ticks)	
Avar-F1	TAAGGACAAGAAGACCCTAAGAAT	*Amblyomma variegatum* (Sumption, K., unpublished result)
Avar-R2	TAACTTCTTCATTAAATAAGAATCC	*Amblyomma variegatum* (Sumption, K., unpublished result)

KEY: R = G or A; H = C, T, or A; M = A or C

CHAPTER THREE.

ISOLATION OF *COWDRIA* FROM DIFFERENT GEOGRAPHICAL LOCATIONS AND VECTORS IN KENYA

3.1 Introduction

Heartwater, is an important disease of cattle, sheep and goats as well as wild ruminants (Uilenberg, 1983), caused by the Rickettsia, *Cowdria ruminantium* (Cowdry, 1926a).

Although the causative organism and vector have long been recognised, Cowdry (1926a & b), problems were encountered in the isolation and demonstration of *Cowdria* (Ilemobade *et al.*, 1975; Ilemobade and Blotkamp, 1978), until tissue culture techniques (Bezuidenhout *et al.*, 1985; Logan *et al.*, 1987), and DNA probes (Waghela *et al.*, 1991; Mahan *et al.*, 1992), for *C. ruminantium* were developed.

The use of susceptible animals remain necessary for isolation of *Cowdria* from the field and workers have used a variety of sources of infective materials. Subinoculation of blood from clinical cases has proven reliable (Du Plessis and Kumm, 1971; Ilemobade, 1976, Jongejan *et al.*, 1984), while sub inoculation of blood from recovered animals has met with variable and generally poor success (Uilenberg, 1971; Du Plessis and Bezuidenhout, 1979). *Amblyomma* ticks are the alternative source of infection for the isolation of *Cowdria* from the field. Adults ticks applied to susceptible hosts (MacKenzie and Van Rooyen, 1981), or inoculated as homogenates (Alexander, 1931; Barré *et al.*, 1984), have enabled *Cowdria* isolates to be made. However, due to the apparently wide range of infection rates in ticks and the possibility of anaphylactic reactions to ground up tick homogenate inoculation, field isolation of *C. ruminantium* is unreliable and requires perseverance particularly where clinical disease is not evident (FAO, 1984).

Information on heartwater in Kenya is scanty (Daubney, 1929-30; Morzaria *et al.*, 1983; Njenga and Mugera, 1989), although the tick vector is widely distributed (Walker, 1974). This chapter describes the successful isolation of *Cowdria* from eight different *Amblyomma* endemic areas in eight districts of Kenya, revealing the wide geographic range of the organism in this country. The relative success of, and problems associated with, the various methods used in the isolation attempts is also indicated.

3.2 Materials and method

3.2.1 ISOLATION SITES

Sixteen isolation sites in twelve different districts were selected within the distribution of *Amblyomma* ticks and thus potential heartwater areas. The location of these is illustrated in Figure 3.1. At a few sites the local veterinary officers reported sporadic suspect heartwater cases, or outbreaks of heartwater were also known to have occurred in the past in these areas.

3.2.2 MATERIALS USED IN ISOLATION ATTEMPTS

3.2.2.1 *BLOOD*

Jugular venous blood from healthy sheep and cattle infested with *Amblyomma* ticks was collected into vacutainer tubes containing Dipotassium ethylene diamine tetracetic acid (EDTA), or Lithium heparin (Becton Dickinson). Blood was kept on ice for 2 to 48 hrs before pooling and inoculation into a susceptible host (Table 3.1). Blood from sheep and cattle was pooled separately.

3.2.2.2 *NYMPHAL TICKS*

Engorged nymphs were collected from cattle from predilection feeding sites for *Amblyomma* nymphs (particularly lower legs and heels), and allowed to moult into adults and harden at 28°C and 85% relative humidity. After hardening they were transferred to an 18°C incubator until needed. After identification adults were applied on backs of susceptible sheep in body patches as described by Heyne *et al.* (1987). Where necessary uninfected male colony ticks were added to facilitate feeding. Table 3.2 gives the details of tick feeds by adults derived from nymphal collections.

3.2.2.3 *ADULT TICKS*

Partially fed *Amblyomma* ticks were removed from cattle and identified. Different *Amblyomma* species were separated and used for isolation by two methods. In the first method, adults were applied to feed in body patches on susceptible sheep (Heyne *et al.*, 1987).

To feed ticks, the backs of sheep were shaved and washed using soap and water.

After drying a calico bag was adhered to wool around the shaved area. Twenty to 60 ticks were secured within each bag in open tubes. After 24 hours the tubes were removed and attached ticks allowed to feed to repletion.

In the second method, tick homogenates were inoculated into the jugular vein in 1.5-2 ml volumes containing approximately 10-tick equivalents in total. Tick homogenates were only prepared from undamaged ticks. Ticks were washed in several changes of water followed by 70% ethanol and then homogenised by grinding in a small volume of buffered lactose peptone (BLP), using a pestle and mortar. Tick debris was removed by spinning at 1,000 rpm for 5 minutes (MSE, Chilspin).The supernatant was adjusted to 4, 10 or 20 tick-equivalents per millilitre with BLP depending on size of collection. Tick homogenate was usually used immediately and injected intravenously into a susceptible sheep which had been pre-treated with 1 ml atropine sulphate (Bimeda, UK), subcutaneously 10 minutes earlier. The remaining homogenate was cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma), and snap frozen in liquid nitrogen.

Tables 3.2 and 3.3 show the details of adult tick feeds and adult tick homogenate inoculations.

3.2.3 EXPERIMENTAL SHEEP

As described in Section 2.2; Corriedale wethers aged between 6 and 8 months from an area non-endemic for *Amblyomma* were used. They were housed in tick-proof barns and maintained on hay supplemented with concentrate and mineral block. Water was given *ad lib*.

3.2.4 MONITORING INFECTIONS

Sheep infected using any of the above methods were monitored as described in Section 2.2.3. They were observed daily and rectal temperature recorded. Clinical signs of depression, anorexia, and nervous signs were looked for. Animals developing severe clinical signs were euthanased on humane grounds although some died suddenly before clinical signs developed.

3.2.5 CONFIRMATION OF INFECTION

The presence *of Cowdria* was confirmed by the examination of brain crush smears made *post mortem* as described by Purchase (1945). The smears were stained with Diff-Quick (Baxter), or Giemsa (Merck), stain and examined for *Cowdria* colonies in the cytoplasm of endothelial cells of brain capillaries. In instances where it was not possible to confirm heartwater in brain crush smears of reacting sheep, intravenous subinoculation of blood stabilate (prepared from that sheep during the pyrexic reaction), was made into a susceptible sheep or goat and allowed confirmation if the animal subsequently developed cowdriosis (Camus and Barré, 1987a).

3.2.6 PREPARATION OF BLOOD STABILATE

When sheep developed fever above 40.5 C, jugular blood was collected into dipotassium EDTA or heparin vacutainer tubes. The blood was chilled to 4 C and undiluted dimethyle sulphoxide (DMSO), added dropwise to 10% v/v while stirring over crushed ice. The blood-DMSO mixture was immediately aliquoted and snap frozen in liquid nitrogen. Two or three initial stabilates were diluted using 20% DMSO in PBS added to make a 50% blood stabilate. The stabilate was dispensed into 2 ml, 3.6 ml, or 4.5 ml cryovial aliquots (Nunc, Roskilde, Denmark or Corning, Quebec, Canada). More than one stabilate was made from the same animal on different days for some of the isolates, but the main one was made on the second day of fever ($\geq$ 40.5°C).

3.3 Results

3.3.1 ISOLATION SITES

Thirteen isolates of *Cowdria* were made from eight sites located in eight different Districts of Kenya as illustrated in Figure 3.1 (map), and summarised in Table 3.4.

3.3.2 TICK SPECIES

Amblyomma variegatum was the sole or predominant *Amblyomma* species found on animals at nine of the attempt sites (includes sites where no isolates were made). *A. gemma* was the predominant species in 4 sites namely, Isiolo, Baragoi, Mariakani and

Bamba. In Maralal *A. variegatum* and *A. gemma* were found in approximately equal numbers. *A. lepidum* was found in just two sites, in Galana in approximately equal numbers with *A. gemma* and in Isiolo. *A cohaerens* was only observed in Transmara. In general, cattle at all sites, which were not subject to tick control had heavy adult tick burdens (sometimes more than 100 per animal), whereas sheep and goats yielded only a few *Amblyomma* ticks as observed by Hoogstral (1956), even when not under tick control and when other ticks, for example, *Rhippicephalus pulchellus* were numerous. Seven of the thirteen *Cowdria* isolates were derived from *A. variegatum*, four from *A. gemma* one from *A. lepidum* and one (Isiolo), was made from a mixture of *A. gemma* and *A. lepidum* (Table 3.4).

3.3.3 ISOLATION METHODS

No isolates were made by subinoculation of pooled blood from suspected heartwater carriers. One out of five attempts was successful by reattachment of adult ticks collected from the field, whilst the feeding of adults derived from nymphs (3 isolates from 5 attempts), or the inoculation of homogenates derived from field adults (9 isolates from 14 attempts), were the more reliable methods for isolation of *Cowdria.* High antibody I.L. were found in cattle (Tables 3.5), at all isolation sites (Table 3.6).

3.4 Discussion

The successful isolation of *C. ruminantium* from eight different districts of Kenya demonstrates the wide distribution of this organism across the country and its presence in ticks infesting healthy cattle sheep and goats. In most of the districts it is likely that a state of endemic stability exists and that sporadic cases or outbreaks occur associated with the introduction of non-indigenous and susceptible animals from non endemic areas, or where transmission of the organism to young animals is irregular due to seasonal tick activity or the use of acaricides (Norval, 1988; Uilenberg, 1990).

Isolation sites were selected on the basis of the presence of *Amblyomma* and in some areas, reports of heartwater cases. At no time during the work were suspect clinical cases of heartwater observed or reported. Therefore isolation of *Cowdria* by

subinoculation of febrile blood, which is considered to be a reliable method of isolation (Du Plessis and Kumm, 1971; Jongejan *et al.*, 1984), could not be attempted. Other workers have obtained clinical cases by grazing susceptible hosts in an endemic area (MacKenzie and Van Rooyen, 1981), but this requires close monitoring and may be impractical. Subinoculation of blood into mice has been used as a means of transporting *Cowdria* from a field case to the laboratory (Haig, 1952). However not all isolates are infective for mice (Uilenberg, 1983; MacKenzie and McHardy, 1987). None of the attempts to isolate *Cowdria* by subinoculation of blood from cattle or small ruminants infested with *Amblyomma* and thus suspected carriers of heartwater (Andrew and Norval, 1989), were successful in spite of the fact that in some cases ticks collected from the same animals did yield isolates. Except in one case, this blood was inoculated 20 or more hours after collection and viability studies have indicated that blood loses significant levels of infectivity by this time (Ilemobade and Blotkamp, 1978; Bezuidenhout, 1984; Logan, 1987). (Norval *et al.*, 1992), observed that needle inoculation of blood was only successful when the donor was high in rickettsia levels, as are considered to occur in clinical cases. It is possible that rickettsemias are low in carrier blood and this combined with loss of infectivity, probably accounts for the lack of success of this method of isolation and it was not used in late attempts.

Field ticks are the obvious alternative choice as a source of *Cowdria.* The use of adults derived from nymphs engorged on cattle proved to be reasonably successful yielding an isolate on three of five attempts in spite of the fact that few of these adults attached and fed successfully, indicating a high infection rate to be present. This method has the advantage of being the most natural, but the disadvantage is the necessity to wait several weeks for ticks to moult into adults and then feed before finding out if an isolation attempt has been successful. The Kiswani isolate (Kocan *et al.*, 1987b), of *C. ruminantium* was obtained from adults derived from *A. variegatum* nymphs collected at the Kenya Coast using this method (A.D. Irvin, personal communication). Collecting adult ticks off animals and feeding them on a susceptible sheep was problematic because many failed to reattach and those that did caused unusually severe irritation making the sheep attempt to remove them; only one of the

five attempts were successful. To avoid these problems, adults collected from the field were inoculated as ground up tick homogenate. This approach has been used previously (Theiler and du Toit, 1928; Alexander, 1931; Bezuidenhout, 1982; Barré *et al.* 1984), and was successful on nine out of fourteen occasions in this work. anaphylactic reaction caused by inoculation of tick homogenate which has been encountered by others (Alexander, 1931; Bezuidenhout, 1987a), was prevented by pre-treatment for it as also done by, Deem *et al.* (1996a).

FAO (198?), rightly concluded that it was by perseverance and luck that isolation of *Cowdria* could be achieved.

Adults picked off animals in the field may be infective, indeed pre-feeding of unfed ticks has been reported to increase their infectivity (Bezuidenhout, 1987a), and this will in effect be achieved in a proportion of the adults collected. However the possible damage to the ticks during removal and their poor reattachment rate makes the use of tick homogenate more likely to be successful than reattaching field ticks. Alternatively, adults collected while feeding may have already shed the infective *Cowdria* that were in their salivary glands, although infective particles may still be in other organs of the same tick (Kocan and Bezuidenhout, 1987). The grinding process used during the preparation of the tick homogenates may liberate these organisms and improve the chances of isolation.

The use of baits to attract and trap unfed *Amblyomma* ticks in the field is also likely to help in future isolations of *Cowdria* (Norval *et al.*, 1987). A. *variegatum* is regarded as the most efficient vector of *Cowdria ruminantium* in Kenya (Walker, 1974), and seven of the isolates made were derived from this species. The four isolates from *A. gemma* and one isolate from *A. lepidum* are the first and second reports (Jongejan *et al.*, 1984), for these species respectively and will be useful in determining if the infectivity of *Cowdria* isolates is highest for the vector species from which it originates. The transtadial transmission of infection in *A. gemma* from nymphs to adults which was shown in this study is also the first report for this species. Since *A. gemma* in particular is widely distributed in drier areas of Kenya and overlaps with *A. variegatum* in drier parts of the laters' range (Walker, 1974), this species is also likely

to play a significant role in the transmission and epidemiology of heartwater in Kenya.

The distribution of *A. variegatum* almost parallels that of *Rhipicephalus appendiculatus*, the vector of *Theileria parva*. With immunisation against East Coast fever (*T. parva* infection), soon to be implemented (Kariuki *et al.*, 1994), the possibility exists that *A. variegatum* and thus heartwater infection, may be reintroduced into areas as tick control is reduced, putting cattle and small ruminants there at risk of disease. The isolates made during this study are solid evidence of the wide distribution of *Cowdria* in Kenya and provide a basis for further studies on this organism and the disease it causes.

Table 3.1: *Cowdria* isolation attempts using pooled field blood.

Isolation site	Susceptible sheep no.	Pool Source	Volume (ml) inoculated	Time to inoculation	Confirmation of *Cowdria*
Galana	228	25 cows	10	48	–
Galana	227	10 cull ewes	10	48	–
Suswa	234	7 cows	10	20	–
Suswa	235	9 sheep	10	20	–
T/Nithi	236	4 goats	10	2	–

Table 3.2: ***Cowdria*** **isolation attempts using adult ticks.**

Isolation site	Animal susceptible sheep no.	Tick species	Number of adult ticks applied (collected as engorged nymphs)	Tick number applied (reattached adults)	Tick numbers attached	Day to fever (°C)		Confirmation of *Cowdria* [+] (days post infection)
						40	41	
Galana	233	Al	18 ff*	n.a.	6	-	16	+[3](17)
Galana	229	Ag[1]	27 ff 30 mm	n.a.	4	20	-	+(22)
Galana	230	Ag[2]	18 ff 20 mm	n.a.	22	-	-	–
Suswa	965	Av	6 ff 1 mm	n.a.	2	10	18	+(19)
Karai	964	Av	3 mm 1 ff	n.a.	1	-	-	–
Karai	237	Av	n.a.	26	3	n.a.	n.a.	–
Mariakani	240	Ag	n.a.	36	nc	n.a.	n.a.	–
Kangundo	241	Av	n.a.	60	11	n.a.	n.a.	–
Bamba	243	Ag	n.a.	40	nc	n.a.	n.a.	+(21)
Marigat	244	Av	n.a.	50	11	n.a.	n.a.	–

KEY * uninfected male colony ticks added to facilitate feeding

Av = A variegatum m = male 1 = nymphs collected from cows n.a. - none/not applicable

Ag = A gemma f = female 2 = nymphs collected from calves

Al = A lepidum nc = not counted 3 = confirmation through subinoculation of blood collected during

Table 3.3: Isolation of *Cowdria* using adult tick homogenates

Isolation site	Susceptible sheep no.	Tick species	Day to fever °C		Confirmation of *Cowdria* [+]
			40	41	
Kathiani	969	Av	11	12	+(17)
Kathiani	972	Av	11	12	+(13)
Kathiani	32	Av	13	15	+(18[1])
Marigat	245	Av	16	17	+(18[1])
Suswa	41	Av	12	-	+(14)
Transmara	966	Av	-	-	–
Isiolo	27	Ag/Al	12	13	+(13[1])
Baragoi	971	Ag	10	13	+(17)
Baragoi	49	Ag	7	9	+(11)
Maralal	970	Av	-	-	–
Busia	967	Av	-	-	–
Karai	242	Av	-	-	–
Asembo bay	52	Av	-	13	+(13)
Holo	51	Av	-	-	–

KEY

+ colonies is detected in brain capillaries

Av = *A variegatum* – - negative

Ag = *A gemma*

Al = *A lepidum*

[1] = sheep euthanised, others died

Table 3.4: Summary of isolation attempts

Site	District	No isolates obtained	Derived from			Isolation methods (successful/attempted)			
			Av	Ag	Al	Adult blood	Adult ex nymph	Adult reattachment	Adult homogenate
Galana	Tana River	2	-	1	1	0/2	2/3	-	-
Suswa	Narok	2	2	-	-	0/2	1/1	-	1/1
Transmara	Narok	0	-	-	-	-	-	-	0/1
Karai	Kiambu	0	-	-	-	-	0/1	0/1	0/1
Mariakani	Kilifi	0	-	-	-	-	-	0/1	-
Bamba	Kilifi	1	-	1	-	-	-	1/1	-
Kangundo	Machakos	0	0	-	-	-	-	0/1	-
Kathiani	Machakos	3	3	-	-	-	-	-	3/3
Marigat	Baringo	1	1	-	-	-	-	0/1	1/1
Baragoi	Samburu	2	-	2	-	-	-	-	2/2
Maralal	Samburu	0	-	-	-	-	-	-	0/1
Busia	Busia	0	-	-	-	-	-	-	0/1
Isiolo	Isiolo	1	-	mixed		-	-	-	1/1
Asembo bay	Siaya	1	1	-	-	-	-	-	1/1
Holo	Kisumu	0	0	-	-	-	-	-	0/1
Tharaka nithi	Meru	0	-			0/1	-	-	-
Total successful attempts		13	7	4	1 (1 mixed)	0/5	3/5	1/5	9/14

KEY Av = *A variegatum* - = not done
Ag = *A gemma*
Al = *A lepidum*

Table 3.5: The serological reactivity of field sera collected at isolation sites to El5 *C ruminantium* (Welgevonden) antigen in c-ELISA test.

Place	Acaricide use	Amblyomma observed	# Sera collected	Number ab +ve	% ab +ve
Galana - cattle (adults)	fortnightly	++	25	17	68
sheep (ewes)	weekly	+	10	9	90
(hoggets)	weekly	+	10	2	20
calves	fortnightly	++	25	13	52
Suswa cattle	sporadic	++	15	15	100
Kiambu - cattle -zebu	sporadic	+	14	4	28.6
- grade	weekly	-	7	0	0
- goats	not done	+	10	8	80
Mariakani - adults	sporadic	+	155	49	31.6
(Kaloleni) - calves	sporadic	+	186	41	22
Bamba	sporadic	++	nd	-	-
Machakos	sporadic	++	12	10	83.3
Marigat	fortnightly	++	14	12	85.7
Maralal -calves	sporadic	+	12	3	25
Baragoi -calves	sporadic	+	42	21	50
Transmara	sporadic	+	nd	-	-
Busia	sporadic	+	nd	-	-
Athi River	weekly	+	nd	-	-
Laikipia	weekly	+	11	1	9.1
Isiolo	sporadic	++	6	6	100
Asembo	sporadic	++	40	30	75
Holo	sporadic	++	nd	-	-

Table 3.6: To show the environs where isolation was attempted, the types of animals sampled and the disease awareness of the local farmers

Place	District	AEZ type	Animal type sampled	Predominant wild life interaction	Heartwater history[1]
Galana	Tana River	low potential (semi arid)	Sahiwal, indigenous cattle and sheep	buffalo, ostrich, large gazelles, small gazelles, elephant wildebeest	A commercial ranch; heartwater disease known to occur and measures being taken to control it. Certain areas on farm avoided.
Suswa	Narok	low/medium potential (semi arid)	indigenous Zebu, some upgraded cattle* and indigenous sheep.	buffalo, ostrich, large gazelles, small gazelles, zebra.	The Narok maasai call the condition 'El milo' They are certain about where the disease occurs and are concerned about the loss it causes.
Karai	Kiambu	medium/low potential	mixed population of Zebu, grade cattle, sheep & goats.	few stray buffalo and gazelles from game parks sometimes	Regular acaricide control practised due to East Coast Fever therefore Heartwater if present unnoticed.
Mariakani	Kilifi	low/medium potential	Mostly from Zebu cattle and few grade cattle. No small ruminants sampled.*	buffalo, small gazelles,	Some acaricide control practised in many herds as ECF big threat to dairying. Clinical heartwater not felt.
Bamba	Kilifi	low/medium potential	Zebu cattle at home steads.	buffalo, small gazelles,	Probably known as one of the (Kilifi) disease syndromes by the local people.
Kangundo & Kathiani	Machakos	low/ medium potential	Large herds of indigenous Zebu Sahiwal crosses at dip site and on farm.	buffalo, ostrich, large gazelles, small gazelles, zebra warthog, wildebeest	Veterinary staff encounter clinical heartwater from time to time. But farmers consider it a lesser problem than ECF.

Table 3.6 (continued)

Place	District	AEZ type	Animal type sampled	Predominant wild life interaction	Heartwater history[1]
Marigat	Baringo	low potential (semi arid)	Indigenous Zebu cattle at dip site	gazelles buffalo bushbuck giraffe, waterbuck eland	ECF considered more important but heartwater known to occur particularly in certain areas by the veterinary staff.
Trans Mara	Narok	low/medium	Indigenous cattle	gazelles, buffalo	Local farmers not aware of heartwater
Isiolo	Isiolo	Medium potential	Indigenous cattle at market place and pasture	buffalo, giraffe, large gazelles, small gazelles, zebra warthog, hippo elephant.	Some farmers not particularly concerned about *Amblyomma* ticks as they do not associate their parasitism on their cattle with disease.
Baragoi	Samburu	low potential	Zebu cattle at homesteads	zebra, giraffe, buffalo gazelles, ostrich	Local people know this disease and make a distinction between it and other cattle diseases.
Asembo	Siaya	medium potential	Zebu cattle in communal grazing	water buck, hippo	Cattle 6 months to a year known to suffer 'a circling disease' from which they die suddenly. But ECF considered the bigger problem.
Holo	Kisumu	medium potential	Zebu cattle at market place	buffalo, ostrich, large gazelles, small gazelles, zebra warthog, hippo wildebeest elephant	Some farmers do not associate any illness to the presence of ticks and are indifferent to the few to moderate numbers of ticks on their cattle

KEY: *- *Bos taurus* (Friesian, Ayrshire,) / *Bos indicus* (African shorthorn Zebu) cattle crosses which produce considerably more milk.
'El milo'- a condition where cattle go around in circles and wanders aimlessly until it dies or is lost and/or mauled by wild animals.
Disease history[1] - this relates only to the farmers with whom discussions were held at the time of isolation

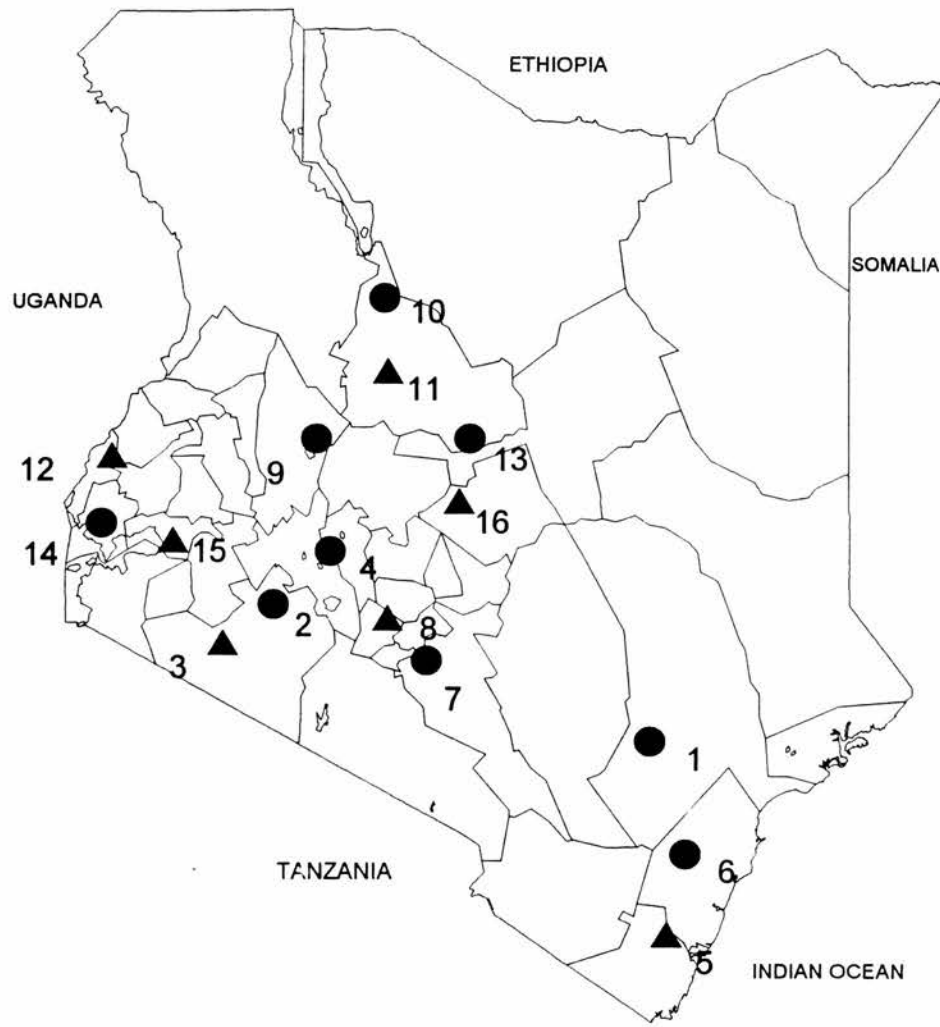

Figure 3.1: Sites of Cowdria isolation attempts in Kenya

● Isolation successful ▲ Isolation not successful

Sites:
1. Galana
2. Suswa
3. Transmara
4. Karai
5. Mariakani
6. Bamba
7. Kangundo
8. Kathiani
9. Marigat
10. Baragoi
11. Maralal
12. Busia
13. Isiolo
14. Asembo Bay
15. Holo
16. T. Nithi

CHAPTER FOUR

INFECTION STUDIES OF KENYAN *COWDRIA* ISOLATES IN SHEEP

4.1 Introduction

Heartwater is a disease that develops rapidly, with clinical signs that are frequently missed and with post mortem lesions that are indistinguishable from other conditions which cause nervous signs and sudden death (Camus *et al.*, 1996). The course of the disease is influenced by the species affected, route of infection and virulence of the isolate and amount of infective material inoculated (Alexander, 1931; Neitz, 1968; Uilenberg, 1983). It is also affected by the age, immune status, and individual or breed susceptibility to the disease (Alexander, 1931; Neitz, 1968). The course of the disease ranges from peracute to inapparent forms (Alexander, 1931; Neitz, 1968; Mare, 1984). Post mortem lesions are very similar in all species (Camus *et al.*, 1996). This includes ruminants and mice (Prozesky, 1987b), as well as wild game (Young and Basson, 1973). However, some post mortem lesions are more common in certain species than others (Steck, 1928; Uilenberg, 1981).

Small ruminants are the species most affected by heartwater. Goats were thought to be the most susceptible (Edington, 1900; Du Plessis *et al.*, 1983), as the peracute form was found commonest in goats and certain breeds of cattle (Henning, 1956, Van Der Merwe, 1979). However, the reaction was reported to be more predictable in sheep (FAO, 1984). Sheep were therefore chosen to carry out these studies because of their commercial farming potential and the high risk they have from this disease in Kenya.

The objectives of this study were to examine the virulence to susceptible sheep, of the different isolates prepared as blood stabilates by the protocol described in Section 3.2.6, of the different Kenyan heartwater isolates. Parameters investigated were clinical manifestations, post-mortem lesions, neutrophil infection rates in cultures established during the febrile period in sheep, and the reaction of recovered sera with *Cowdria* antigen in Western blots. The detection of *Cowdria* in the blood of pyretic sheep by PCR was used as an additional confirmatory test.

4.2 Materials and Methods

Clinical, pathological and post mortem lesions associated with infection with 11 isolates in Corriedale sheep was recorded and are discussed in this chapter. The infections were initiated as part of several experiments being

(1) stabilate testing to establish viability of stabilates.

(2) cross immunity trials (Chapter 5).

(3) tick infection experiments (Chapter 7).

4.2.1 SHEEP

These were susceptible sheep of the Corriedale breed as described in Section 2.2 and the plan of how they were used and numbers are shown below in Table .4.1. A plan of the sheep used in the cross immunity trial is shown in Chapter 5.

4.2.2 *COWDRIA*

Eleven isolates from 8 districts in Kenya as shown below in Table 4.1. Ten were newly isolated and the eleventh, the Kiswani isolate, was provided by Dr. Morzaria of ILRI. All were blood stabilates, made on the second day after fever of 41°C (Section 3.2.6.), for the purpose of this work, except as indicated. The zero passage (i.e. blood collected from sheep of isolation), of the isolate was used except in the case of Bamba and Marigat stabilates where the first passage due to unavoidable circumstances.

4.2.3 *MODUS OPERANDI*

Prior to studies on cross immunity and tick infectivity, the viability of stabilates were established by infection in a pair of susceptible sheep. One millilitre of stabilate was inoculated into the jugular vein of each sheep with exception of sheep nos. 238 and 239, which were inoculated with 7 ml of 50% stabilate of Galana 229, and all control sheep used during homologous boosting challenge in the cross immunity trials in which sheep were inoculated with two millilitre of blood stabilate diluted 1:6 (= 1/3 ml neat equivalent diluted in RPMI1640 medium). The sheep were then monitored for the infection as described in Section 2.2.3.

4.2.4 CLINICAL AND POST-MORTEM INVESTIGATION

Sheep were monitored as described in Section 2.2.3. The following clinical parameters were determined:- incubation, period until rectal temperature reached 40°C, incubation, period until temperature reached 41°C, maximum temperature reached, duration of fever and days to death or recovery. The sheep were also checked daily by author and findings recorded on a clinical record, for the following clinical signs: expression and behaviour, anorexia, weakness, rapid or shallow breathing, incoordination, diarrhoea and any other observable changes using a check list (Appendices 4.1a & b). Sheep *in extremis* were euthanased promptly (unnecessary suffering was avoided), examined post mortem by the author and findings recorded on a post mortem record (Appendix 4.2), for each animal.

4.2.5 SEROLOGY

One sheep in the stabilate testing pair for all eleven isolates, was deliberately treated on the second day of fever (≥ 40.5°C), using 20 mg/Kg body weight with long acting tetracycline (Tetroxy LA, Bimeda, UK), in order to obtain serum post recovery. Serum was collected pre infection (all sheep), and between 28 and 35 days pi. from the sheep that were treated or those that made a natural recovery, and tested for antibodies against *C. ruminantium* (Welgevonden), antigen in the Competitive ELISA described in Section 2.6.1. A titration using the cELISA test was performed with the sera that were also used for testing in Western blots. The titre was defined as the highest dilution that gave an inhibition of > 50%.

4.2.6 DETERMINATION OF NEUTROPHIL INFECTION RATE

The methods employed in the culture and sampling of *Cowdria* in neutrophils have been described in Section 2.8.1. Sheep infected with 11 *Cowdria* isolates (ten newly isolated and one donated), from different parts of Kenya were studied. The core of this study was based on sheep used for stabilate viability testing (all 11 isolates), and animals used for *Cowdria* isolation (five isolates, Table 4.1), amounting to 2 to 4 febrile sheep for each isolate in this part of the study. More neutrophil cultures were established from cross immunity and tick infectivity sheep. Except for sheep in the

cross immunity trials and tick infectivity studies, neutrophil cultures were established daily from blood for the duration of fever. Cultures were initiated as soon as the sheep were deemed to be reacting to heartwater (having a fever (40°C), and stopped when temperature returned to normal (< 40°C), or following treatment with oxytetracycline (Appendix 4.3). Some immunised sheep (therefore not in the primary infection), and control sheep were sampled randomly when they were reacting with high fever (≥ 40.5°C), as an expansion of the neutrophil study.

Cytospin smears were made from each culture flask daily from the day it was prepared until the culture was discarded on the third to fifth day.

Distinct azurophilic colonies were sought in microscopic examination of Giemsa stained cytospin smears: single bodies (single elementary bodies), were indiscernible and their presence could therefore not be determined. The character of the morulae was noted and the infection rate estimated by counting the morulae (colonies), in five fields containing 100 - 150 neutrophils each and taking the average, or counting infection rate in four hundred neutrophils. The whole smear was examined for morulae if no infection was observed in 400 neutrophils.

4.2.7 ISOLATION OF *COWDRIA* IN ENDOTHELIAL CELL CULTURES FROM THE BLOOD OF INFECTED SHEEP

The methods employed in the attempts to establish *Cowdria* in endothelial cell culture have been described in Section 2.7.2. This work was carried out by Joseph Wafula of the TBD project at the National Veterinary Research Centre, Muguga in Kenya. Blood was collected in Lithium Heparin tubes from most of the febrile sheep reacting to heartwater infection throughout the course of these studies in an attempt to isolate the 10 new *Cowdria* isolates in culture. The Kiswani isolate had already been established in culture (Byrom *et al.*, 1991). Blood was collected once, twice or three times from a sheep during the pyretic period only at very high fever(≥ 40.5°C). Animals were not sampled during atypical pyrexia (< 40.5°C), or after an animal had been treated with Oxytetracycline. The cultures were assessed by examining the flask for cytopathic effect, and making cytospin smears of the supernatant. The smears

were stained with Giemsa (5% for 40 minutes or 10% for 20 minutes), and examined under the light microscope (Leitz, Leibovitz, Germany), under x 50 then x 100 magnification and examined for the presence of elementary bodies.

4.2.8 DETECTION OF *COWDRIA* DNA IN THE BLOOD OF PYRETIC SHEEP USING THE HE1(cr)/HE3(l) PRIMERS.

This test was performed on these new isolates to demonstrate that an agent was present in blood whose genome was amplifiable with PCR primers specific to *C. ruminantium*. The HE1(cr)/HE3(l) primers (Watson, 1993), were employed and have been described in Section 2.8. These primers amplify a 388 bp product in the 16S rRNA gene of *Cowdria*. Blood samples tested had been collected during the pyretic period only.

A full description is given in Section 2.8. Briefly, samples were denatured at 94°C for 10 minutes, then amplified for forty cycles of sixty seconds at 94°C for denaturing, sixty seconds at 65°C for annealing and ninety seconds at 72°C for extension. Amplified products were visualised on a 1.5% agarose gel in 1×TBE containing 0.2µg/ml ethidium bromide.

4.2.9 WESTERN BLOT ANALYSIS OF ANTIGENS RECOGNISED BY SERUM FROM HEARTWATER RECOVERED ANIMALS

The purpose of Western blotting was to determine if the antibody response to infection with these agents was typical of *C. ruminantium* infection. Sera of sheep infected with the different isolates were reacted with antigens of the Welgevonden stock to examine the antigens recognised. Sera tested by western blot analysis described in Section 2.6., were collected prior to infection and after recovery, between day 28 and 35 post infection, from isolation or first stabilate infectivity testing.

4.2.10 THE DIAGNOSTIC INDEX

This was a factor calculated on colonies in the smears of sheep brains infected with a particular isolate, to express the ease with which they were detectable under the

microscope for that isolate. The ease (or difficulty), of finding a colony(s) at different infection rates under microscopic examination, in smears of sheep brain stained with Giemsa was assessed. The percent infection rate was classified into four levels of detection categories according to the relative ease of detection. Viz.: the first level - no colonies seen, considered very difficult or not possible to diagnose; second level - less than one percent endothelial cell infection rate, considered difficult to diagnose; third level -between 1% and 4.9% considered to be easy to diagnose; and the fourth level - equal to or greater than 5%, considered to be very easy to diagnose. A score (0, 2, 5 and 10 respectively), was awarded for the ascending levels of infection categories (Tables 4.4a & b). The infection rates of animals infected with an isolate were categorised into the different levels. The mean of the score of all the animals infected with an isolate was calculated and converted into a whole number, (i.e. multiplied by 10) and taken to be the diagnostic index (DI), for that isolate. The DI was used to compare the ease of microscopic diagnosis for the different isolates.

4.3 Results

4.3.1 CLINICAL MANIFESTATIONS OF COWDRIOSIS IN SHEEP

4.3.1.1 INFECTION

Inoculation of blood stabilate (one and seven millilitres), into the jugular vein of susceptible sheep resulted in *Cowdria* infection which was reproduced with 11 stabilates representing the 11 isolates. It was noted that all sheep except 3 out of 72 (4.2%), became infected after the first inoculation.These 3, (nos. 102, 239 & 61) (Table 4.1), were negative clinically and serologically but reinoculation of these sheep led to heartwater disease. The establishment of the agent in the sheep was detected by various methods and the clinical and pathological signs are described below.

4.3.1.2 INCUBATION

Incubation period varied between the stabilates but tended to be similar for a particular stabilate (Table 4.2a & 4.2b). The median time taken from inoculation to reach a fever of 40.0°C was 12 days (range 7 to 19 days), for the various stabilates, in 42 sheep, ones that received 1ml neat stabilate (Table 4.2a). Thirteen sheep had a temperature rise directly to 41°C, median time taken 10 days (range 8 to 18 days pi). The overall median number of days to fever (40°C or 41°C), was 11 days. Statistically, incubation period of the 11 isolates fell into two overlapping groups that were significantly different from each other ($p < 0.01$, KW statistics), but members of a group were not significantly different from each other. The first group comprised the isolates with median incubation ranging from 8 to 12 days, viz.: Suswa (8), Baragoi (9), Asembo (10), Kathiani 972 (10), Kiswani (11), Gal 233 (12), and Isiolo (12). Together they had a median of 10 days incubation. The second group comprised the isolates with a median incubation ranging from 10 to 16.5 days: viz.: Asembo (10); Kathiani 972 (10); Kiswani (11); Galana 233 (12); and Isiolo (12); Kathiani 32 (13); Galana 229 (13.5); Marigat (14) and Bamba (16.5). Together the second group had a median incubation of 12 days.

4.3.1.3 PYREXIA

A rise in fever, cut off level of 40°C was used. All sheep became pyrexic during the infection. The fever usually reached 40°C first in some animals whereas it was first detected at 41°C in some sheep. In the majority of sheep fever quickly rose from 40°C to 41°C in one to three days. The maximum temperature for most animals was well over 41°C and reached 42°C in a few animals. Fever remained below 41°C through out the infection in some sheep. The median peak fever overall was 41.7°C and ranged from 40.9°C with Kiswani infection to 41.9°C with Asembo infection (Table 4.2a).

4.3.1.4 COURSE AND DURATION OF DISEASE

The median time taken from inoculation to death in the sheep infected with the different isolates was 13 days (range 9 to 22 days), but each stabilate had a slightly

different median number of days to death (median days to death ranged from 10 days to 20 days for all isolates) (Table 4.2a) (p=0.0017 considered to be very significant, KW statistics). The duration of disease from the time of hyperthermia to its conclusion, lasted a median of 5 days (range 1 to 15 days) (p=0.02 which is considered significant, KW statistics). Sheep died after a median of 4 days (range 1 to 8 days of fever), sheep that recovered were febrile for a median of 7 days (range 3 to 15 days), before the temperature returned to normal. They recovered (i.e. temperature returned to normal) on a median number of 23 days (range 16 and 26 pi (median to recovery for isolates separately 18 to 25 days). The overall median number of days of fever for all sheep was 6 days. There was a positive correlation between chronic infections and natural recovery, and acute disease and death.

4.3.1.5 CLINICAL SIGNS

The proportion of animals exhibiting particular clinical signs is given in Table 4.3. Pyrexia was the first manifestation of heartwater in infected sheep. Apart from fever, clinical signs were primarily respiratory and nervous. Respiratory signs occurred a day to a few days after hyperthermia, although in a few sheep, they coincided with the fever rise. When sheep were treated on the second day of fever, increased respiration for one day was the only clinical sign in 12 out of 96 (12.5%), clinical signs were not seen in the other 84 sheep.

4.3.1.6 MORTALITY RATE

The median mortality rate of untreated sheep infected with the different isolates was 100% (range 0% to 100%) (Table 4.2a). Seven isolates killed all untreated sheep: Suswa (n=5), Baragoi (n=6), Kathiani 972 (n=5), Kiswani (n=2), Galana 229 (n=3), Galana 233 (n=1), and the Isiolo (n=1), whereas a proportion of the untreated sheep survived infection with four isolates, Marigat (1 out of 3), Asembo (3 out of 7), Bamba (7 out of 8), and Kathiani 32 (2 out of 2). There was no mortality in the sheep infected with different isolates treated with oxytetracycline in order to provide recovered serum (Table 4.2b in bold). The difference in mortality between the mild isolates and the virulent isolates as groups was extremely significant ($p < 0.0001$),

Fisher's exact test. The breakdown of the contribution to this significance from different isolates is given in Appendix 4.7.

4.3.1.7 VIRULENCE OF ISOLATES

The virulence of the isolates was evaluated by a reaction index (Du Plessis, 1985) (Section 2.2.3.1). There was a spectrum of virulence among the isolates from highly virulent, to moderate and low virulence (Tables 4.2a, b and 4.3b & c). The majority were highly virulent. The median reaction index was 47.84. The order of reducing virulence (median RI values, Tables 4.2a), was Suswa (51.8), Baragoi (50.1), Kathiani 972 (49.6), Kiswani (46.9), Galana 229 (46.2), Marigat (47.7), Asembo (45.00), Bamba (12.5), and Kathiani 32 (11.0). Two isolates had one animal representing each of them, Galana 233 (RI = 48.8), and Isiolo (RI = 47.8).

4.3.1.8 BRAIN CRUSH SMEARS

Observation of colonies in brain crush smears was the standard means of confirmation of all *Cowdria* infections that resulted in death. Infection rate in endothelial cells were determined and a median of 5.8% (range 0-16.8%), was found. A diagnostic index (DI), was formulated (Section 4.2.10), to take into consideration the number of animals with different levels of colonies in the endothelial cells of the brain capillaries, Tables 4.4a,& b. The diagnostic indices were separated into those due to primary infection or those of deaths resulting from heterologous challenge infection in order to observe if detectability was affected by the previous infection. The median diagnostic index was 42.2 (n = 9) (range zero (n = 3), to 91.7 (n = 6) (Table 4.4a). The inclussion of challenged immune animals caused the diagnostic indices to alter to an extent; Kathiani 972 infections dropped from 91.7 to 80.6, and Suswa infections from 36.5 to 35.2 while that for Kiswani infection increased from 30.0 to 47.5%. It was notable that colonies could not be detected in some sheep infected with Bamba (2 out of 2), Kathiani 32 (1 out of 1), Marigat (1 out of 3), and Suswa (2 out of 31), and that the DI of the Suswa isolate which is highly virulent (median RI = 51.8), was conversely low (DI = 35.2), resulting in difficulty in confirming the disease.

4.3.2 POST MORTEM LESIONS

There was typical heartwater post mortem lesions with all isolates. The lesions from all animals that died or were euthanased during isolation, infectivity testing, cross immunity trials during this study were included and are summarised in Table 4.5 and Appendix 4.4. There were eighty nine sheep that died or were euthanased due to cowdriosis. Most organs were affected to various degrees in different sheep. The most frequently affected organs were the vital organs; kidneys (93.3%), lungs (91.0%), liver (86.5%), and heart (77.5%),. Most sheep (79.8%), had hydrothorax and or hydropericardium (71.9%), but 8.9% of the sheep had neither (Appendix 4.4). The gall bladder was full and 67.9% of the 91.0% sheep with gall bladder lesion appeared to have leaked onto adjacent internal organs. The omental fat looked bright yellow with bile when seen in fresh carcasses (Figure 4.4), or the internal organs appeared to be decomposed far beyond the expectation that duration after death would have caused. Another notable feature was perineal soiling as evidence of terminal diarrhoea; 89.5% of perineal soiling were Suswa (n = 17), infections. One Baragoi and one Kiswani infected sheep also had manifestation of diarrhoea. None of the sheep infected with the other isolates displayed symptoms of diarrhoea. (Appendix 4.4), Ascites was present in 32.6% of the sheep.

4.3.3 NEUTROPHIL INFECTION RATE

The results of *in vitro* culture to determine the infection rate in neutrophils is given in Table 4.6. Neutrophil infections which can be seen in greater detail in Appendix 4.3. With six of the isolates, more than 50% of the cultures developed infected neutrophils. In the other five isolates, less than 30% of the cultures developed infected neutrophils. The greatest extreme was between Galana 229 in which morulae developed in 17% of cultures (n = 6), and Kiswani in which nearly 86% of the cultures (n = 7), developed morulae. Infection rates were rarely greater than one percent. This is further supported by the cultures initiated from sheep in the cross immunity and tick infectivity studies in which 13 out of 59 (22.03%), animals, one culture (two to three were made for at least 50% of animals), developed greater than

1% infection rate. (Appendix 4.5). The results support the classification into high, intermediate and low infection rate. There was a regression of RI on neutrophil infection rate, $0.10 > p > 0.05$ i.e. trend towards significance.

4.3.4 ISOLATION OF *COWDRIA* IN ENDOTHELIAL CELL CULTURES FROM BLOOD OF INFECTED SHEEP

Out of attempts from 3 to 27 infected sheep per isolate to grow *Cowdria* in endothelial cell culture from the different isolates, only four out of the 10 isolates were successfully established (Table 4.7). Suswa, Baragoi and Kathiani 972 culture were established from two to four sheep each. Isiolo culture was established from one sheep infected with Isiolo blood stabilate in Harare, by Mrs Gillian Smith of the SADC heartwater project. The other isolates have yet to be established in endothelial culture. Elementary bodies were seen starting from day 19 earliest to 49 in the different isolates in our laboratory, but Mrs Smith found elementary bodies on days 13, 15, 17, 17 and 20 in the five cultures inoculated on the same day with plasma obtained on the third day of febrile reaction in the infected sheep.

4.3.5 DETECTION OF *COWDRIA* ISOLATES IN FEBRILE ANIMALS DURING STABILATE TESTING BY PCR AMPLIFICATION USING THE HE1(cr)/HE3(l) PRIMERS

The preparation of the *Cowdria* DNA from blood, the amplification and the detection of products by agarose gel electrophoresis have been described in Section 2.2.4, and 2.8.

Amplification of the DNA extracts using HE1(cr)/HE3(l), resulted in amplification of the expected 388 bp. A product from DNA samples of all isolates prepared from febrile blood directly or neutrophil extracts from cultures that were being prepared concurrently (Figures 4.2a - d).

4.3.6 WESTERN BLOTTING

Sera from the recovered sheep recognised the immunoreactive surface antigens on Western blotting of *Cowdria ruminantium* (Welgevonden) antigens. These included

the immunodominant 32 kDa antigen that was recognised by all sera but also 21, 27, 40, 46, 58 and, 85 kDa proteins. There were sera that recognised very few antigens and others that recognised numerous antigens (Figures 4.3a - d). Different sheep infected with the same isolate detected similar but not the same number of antigens. All sera recognised the 32 kDa and also several other antigens. The 32 kDa region was always immunodominant (very thick). Antigens larger than 90 kDa, were less immunodominant (weakly recognised), in the blots.

4.3.7 SEROLOGY

The pre infection sera of the sheep which had a low antibody titre to the EL5 *Cowdria ruminantium* (Welgevonden) antigen, were below the 70% cut off inhibition level adopted in the competitive Enzyme Linked ImmunoSorbent Assay (cELISA), test. All recovered sera (neat), for each isolate, competed strongly in the (cELISA), test and had levels well above the cut off level of 70%, frequently 90% inhibition (Appendix 4.6a-c). Post infection serum used for Western blotting had cELISA titre between 1:2 and 1:128 (Figures 4.1, 4.3a-d). Interestingly the median titre in sheep that were treated was 1:4 compared with 1:32 in sheep that recovered naturally.

4.4 Discussion

4.4.1 CLINICAL MANIFESTATIONS OF COWDRIOSIS IN SHEEP

4.4.1.1 INFECTION TAKE

Heartwater disease was reproducible by jugular vein inoculation with one millilitre of blood stabilate with all the isolates (Table 4.2a), (and with other volumes as well (Table 4.2b)). However i/v inoculation failed to induce heartwater infection the first time in three sheep. It is only in cattle that a significant proportion of animals have been reported to fail to react to known virulent infection, (Du Plessis *et al.*, 1984). The author believes factors other than human error were responsible for failure to cause infection as it occurred again during immunisation (sheep # 7, Appendix 5.1), with the same (Bamba), isolate. It is concievable that the Bamba stabilate could have fewer than adequate number of organsms in the inoculum to cause infection in all animals, due to a lower multiplication rate of this agent compared to the other

isolates. Hence it is necessary to titrate stabilates to establish the ID^{50} of the different stabilates. Failure for infection to establish has led to the modification of the infection method (during heartwater vaccination), by inoculating animals twice 5-7 days apart, (Van Der Merwe, 1979), perhaps to reduce the element and suspicion of human error.

4.4.1.2 INCUBATION

Incubation is influenced by the species affected, virulence of the isolate and amount of infective material (Alexander, 1931; Neitz, 1968; Uilenberg, 1983). Du Plessis and Malan (1987d), noted that infectivity of different batches of blood vaccine, may be different and may result in variation in incubation period.

There was a spectrum of variation in incubation period between the representative stabilate of isolates used in this study. According to Neitz (1968), the incubation ranges between 1-3 weeks after intravenous inoculation in small ruminants. It was the case for the majority of the isolates in Corriedale sheep in this study. Gruss (1987), found similar incubation for blood stabilates in naive goats infected with Ball 3 vaccine, as did Jongejan and Wassink (1991) with four isolates and Martinez *et al.* (1994), with the Gardel also in goats. Cattle had much longer incubations averaging 18 days (Neitz, 1968; Lawrence *et al.*, 1995). This is in agreement that more susceptible animals get a shorter incubation period. It is also possible that particularly between the isolates some of the differences in incubation was due to the amount of organisms in the stabilates being different as titration was not done. The sheep that were infected with 7 to 20 fold higher volumes certainly had incubation periods that were on the lower scale for those isolates. However this may not be significant with blood or blood stabilate as there were sheep infected using one third of a millilitre of blood equivalent that had the same or shorter incubations than those inoculated larger volumes (Table 4.2b).

4.4.1.3 PYREXIA

Symptoms of heartwater vary, but usually begin with fever and may involve neurological signs and respiratory distress. Clinical diagnosis is based on symptoms, Yunker (1996).

As found by Uilenberg (1983), hyperthermia usually developed suddenly without overt clinical signs. It was characteristically high (≥ 41°C), with small fluctuations, but in a number of sheep it was bi- or multi-phasic. In a minority of sheep hyperthermia was marginal (≥ 40°C), and developed more gradually creating an impression that the heartwater infection was not serious or critical. These animal sometimes died suddenly and heartwater was confirmed retrospectively at autopsy and microscopically. This atypical behaviour was more characteristic and dangerous in the Suswa infected sheep. It was deceptive because a high fever (approaching 41°C), is what is associated with heartwater and a low fever (around 40°C), associated with possible concurrent infection with for example *Anaplasma* (Neitz, 1939). It was also the case with this isolate that after fever the disease was little tolerated in the sheep leading to the out come in Table 4.3c, but this fact did not become evident when the isolate was first stabilate tested. A subnormal drop in fever occurred just before death in a few animals as found by Alexander, (1931) and Uilenberg, (1981, 1983).

4.4.1.4 COURSE AND DURATION OF DISEASE

The course of the disease ranges from peracute to inapparent (Alexander, 1931; Neitz, 1968; Mare, 1984 Yunker, 1996), meaning the disease lasts from a few hours to 36 to 48 hours or never becomes evident. Between the isolates, there was a lot of variation in the course and duration of the disease. After the onset of fever, in the most virulent, death swiftly intervened: for the Suswa isolate, which was the most virulent, the median duration of illness before death was three days. The other isolates had longer courses associated with an attendant fever and other clinical signs. Most people recognise the infection when the respiratory signs set in which, in this study (which being experimental animals were closely monitored in contrast to the field situation), were found to occur after two days of fever in the majority of animals. The fever itself would in all probability be missed upto the point when the signs appeared. This is supported by the fact that Cassard (1961), was unable to treat successfully any of twelve Friesians infected naturally. This is a pointer of a possibly similar outcome in the event of a tick transmitted outbreak of Kenyan cowdriosis.

4.4.1.5 CLINICAL SIGNS

The new isolates behaved in sheep, like other known *Cowdria,* as reviewed by Van De Pypekamp and Prozesky (1987). There was some differences between isolates in the disease resulting from infection with blood stabilate inoculated intravenously into susceptible Corriedale sheep. At first animals were clinically normal but gradually become anorexic and ultimately stopped feeding. Nervous symptoms ranging from mild incoordination to convulsions occurred in some of the sheep as found by Alexander (1931). At the top RI ranks, disease developed rapidly and with few presenting clinical signs, frequently nervous or gastrointestinal symptoms occurred terminally (Uilenberg, 1971). Occasionally animals remained standing with their heads held low or pushing against objects.

At the lower spectrum, many sheep remained responsive and alert throughout the course of the disease and displayed only accelerated breathing and apathy. This was seen mostly in infections with the Kathiani 32, Bamba, Marigat, and Asembo isolates considered to be the milder isolates. Many recovered naturally without treatment. The isolates could not be distinguished on clinical signs but diarrhoea was commonest among the Suswa infected sheep, though seen in one animal each infected with the Kiswani and Baragoi isolates. Similarly, nervous signs were most severe in the Baragoi infected sheep though also seen in some Suswa, Kathiani 972, Galana 229 and Asembo infected sheep.

4.4.1.6 VIRULENCE OF ISOLATES

The reaction index (RI) (Du Plessis, 1985), was useful in imputing an order of virulence among the Kenyan isolates. It was possible to rank the isolates from Suswa, the most virulent (median RI = 51.78), to Kathiani 32, the least virulent (median RI = 9.26). (Table 4.2a). High (Du Plessis, 1984b), and low virulent (Du Plessis *et al.*, 1992b), *Cowdria* for sheep have been previously reported but the high virulence *Cowdria* are more numerous. It was surprising that the Asembo isolate was moderate (67% fatal) and the Bamba isolate mild (12.5% fatal) during stabilate testing whereas they killed the sheep used in isolation rather precipitously. This showed that they

could be mild or severe in individual sheep. The Kathiani 32 isolate was non lethal (Section 4.4.1.6). (In retrospect, the Kathiani 32 isolate animal may have recovered naturally if it had been observed slightly longer). Sheep belong to a highly susceptible ruminant species (Uilenberg, 1983; Yunker, 1996), and the Corriedales used were of a susceptible age compared to young animals in which mild and inapparent disease prevail (Uilenberg, 1981; Yunker, 1996). In this study, the reaction index was a measure of virulence as reflected in the total fatalities in seven of the eleven isolates and declining down the isolates, only one isolate was not fatal at all. The Kathiani 32 was regarded to be a *Cowdria* because apart from already mentioned *Cowdria* characteristics, it was also isolated from *Amblyomma* ticks and it infected sheep with a characteristic high fever and respiratory embarrassment like the other *Cowdria.* At this juncture, lack of virulence has been arrived at after testing in relatively few animals and appears to be the only main difference Kathiani 32 has from the other isolates. Further testing is required to conclude total lack of virulence, although this being the case, its detection by PCR and reaction in western blots, would affiliate it with the other *Cowdria* until sufficient proof called for it to be reclassified. Allsopp and Visser (1994), found an unexpected degree of heterogeneity in the sequence of a part of the srRNA gene.

4.4.1.7 MORTALITY RATE

The design of the study unavoidably resulted in an unequal testing of different isolates. Nonetheless, in the animals tested in this study (Table 4.2a), the mortality rate was 100% for 7 isolates while four isolates caused lower mortality. More accurate mortality rates need to be obtained for isolates where few animals were used. Differing mortalities (in susceptible hosts), for different *Cowdria* isolates is in agreement with mortalities for isolates found in other parts of Africa and the Caribbean, Uilenberg (1983). For instant 40% mortality was found in sheep with the Kumm stabilate (Du Plessis, 1982). It was significant that there was mortality of (60%), with Suswa infection and (5%), in Baragoi infection before and inspite of standard treatment with oxytetracyclines (Tables 4.1, and 4.2c) (experienced during immunisation (Chapter 5)). There was no mortality after similar treatment in the

Asembo or Bamba infection. One isolate, Kathiani 32, did not result in mortality and its identity as a *Cowdria* may be questioned. However, beside its similarity with the other *Cowdria* in amplifying a product of 388 bp (Figure 4.2c), in the PCR with the HE primers, morulae were seen in the neutrophils, and recovered sheep sera recognised very similar antigens to those recognised by the sera from other *Cowdria* infections especially its virulent sister Kathiani 972. (The two Kathiani isolates were rather parallel in their different behaviour to the Mara isolates made by Du Plessis *et al.* (1992b), on one farm). Further investigation need to be made on Kathiani 32 isolate because it may have a mortality rate that was not detected at the level it was tested, and it signals that there are therefore other mild *Cowdria* in the field.

4.4.2 POST MORTEM LESIONS

The pathology found in heartwater has been reviewed by Prozesky (1987b), and the expected pathology was encountered in this study. Unequal testing of the different isolates (which was unavoidable), resulted in very few specimens being available for some isolates therefore pathology of heartwater in sheep was compiled for the whole study for better comparison and discussion in this chapter.

The eighty nine sheep that died or were euthanased due to cowdriosis, were primarily associated with three isolates: the Suswa, Baragoi and Kathiani 972 isolates. Post mortem pathology for all isolates was suggestive of heartwater because of the increased fluids in body cavities (Table 4.5 and Appendix 4.4), as found by Steck (1928), and Uilenberg (1981), and reviewed by Uilenberg (1983), and Prozesky (1987b). However the variation in the proportion of the lesions seen between isolates was not significant. For example the gall bladder lesion analysed using the Kruskal-Wallis Statistics ($p = 0.1935$), was not significantly different between the isolates. However, some post mortem changes in this study were more common to certain isolates than to others compared to the findings of (Steck, 1928; Uilenberg, 1981), who found some changes more common in certain host species than others. The finding of diarrhoea in the Suswa infections compared to other isolates was highly significant ($p < 0.001$).

Hydrothorax and hydropericardium were frequent but sometimes absent (but colonies were found in all of these animals). In contrast to Prozesky (1987b), the commonest pathological finding was a full gall bladder which was turgid (very full), and in many animals appeared to have leaked some bile, (indicated by 4+ in Appendix 4.5), on to adjacent internal organs. This is the first report of internal organs being stained yellow due to 'bile leakage' (Figure 4.4).

The lungs were usually a normal colour, incollapsible, with 'veins' of mild emphysema and interstitial oedema and sometimes patches of hepatization in the apical lobes. The liver was moderately enlarged, stained and/or slightly jaundiced and/or necrosed. The kidneys had different degrees of pallor even cooked appearance, with different degrees of subcortical haemorrhage. The spleen was usually slightly affected but could be pulpy. Some sheep had ascites, and petechiae of various mucosal surfaces including the intestines and the conjunctivae, all findings that have been found by the authors as reviewed by Prozesky (1987b). Some sheep also exhibited subdural haemorrhage, congestion or oedema as found by Van de Pypekamp and Prozesky (1987) (Table 4.5 and Appendix 4.4).

4.4.3 HEARTWATER CONFIRMATION

4.4.3.1 BRAIN CRUSH

Definitive diagnosis of heartwater is only by observation of colonies in the cytoplasm of brain capillary endothelium (Uilenberg, 1983; Yunker, 1996). Biopsy smears from the brain served to confirm heartwater in all but a few individual animals. Cowdry (1925b), found that unless samples were taken soon after death (<6 hours), *Cowdria* lost their staining characteristics and the author supports this to hold true upto the present in some field situations. However, Uilenberg (1971), and Camus and Barré (1988a), found post mortem diagnosis was still possible after a much longer period if the brain was removed from the cranium and kept at various temperatures: room, refrigerator or frozen at -31°C and the author experimented with refrigeration and found this also to hold true. Although brain crush smears were made immediately and never more than 10 hours after death in this study, there was a paucity of colonies which was not related to delayed preparation of brain smears in the Kathiani 32, and

Marigat infections (Tables 4.4a, b). This scarcity may be related to their lower virulence because the Kathiani 32 and Marigat carcasses appeared relatively fresh. However, both Bamba deaths (Table 4.5, Appendix 4.4), were associated with destructive haemorrhagic lesions which could have concealed a few colonies that may have been present in the brain. Albeit the Suswa isolate was highly virulent (Table 4.2a & 4.3c), few colonies too were found in the brain smear of the majority of animals (low DI, Table 4.4a, & b). In these cases it would profit to look for evidence of the organisms perhaps by an immunohistochemical technique of staining tissues (Jardine *et al.*, 1995), or testing for presence of *Cowdria* DNA (Peter *et al.*, 1995), in a laboratory with adequate facilities available. The author is of the opinion that with all due care, definitive diagnosis may not be possible in all cases of heartwater. In this study around 7% of animals were found to be impossible to diagnose (i.e. no colonies). The author considers the diagnosis of field cases (in Kenya), in ordinary diagnostic laboratories, examining Giemsa stained brain crush smears, to be confirmable in at least 60% of heartwater cases with ease (levels 3 and 4, Tables 4.4a & b), according to findings of this study.

4.4.3.2 NEUTROPHIL CULTURE

The ability of morulae to develop in the neutrophils of animals infected with an isolate was correlated to the median RI of that isolate. The correlation extended to an individual sheep having the highest RI within a group, and was most likely, the sheep with the most highly infected neutrophils among the sheep infected with that isolate. This interprets to low virulence isolates also having less frequent and lower infection rates (which may be the source of low infection rates reported by Jongejan *et al.* (1989), than the virulent isolates with a correlation that was approaching significance ($0.1 < p > 0.05$). Presence of morulae in neutrophils permitted the confirmation of diagnosis of cowdriosis in retrospect, albeit with difficulty especially because a diagnosis was virtually needed in the low virulence isolates. Morulae in neutrophils of individuals in which brain colonies were not seen, were identified as being *Cowdria* by virtue of brain colonies present in other sheep infected with the same isolate. Suswa morulae developed relatively frequently in neutrophil cultures. (Table 4.6 and

Appendices 4.3 & 4.5). and this was the only isolate that had proportionately higher infection rate in the neutrophils in comparison to colonies in the brain capillary endothelium. The Kathiani 972 and Baragoi isolates were "classical" *Cowdria* with numerous colonies in neutrophil cultures (and in the brain) (Appendix 4.3), in agreement with the findings of Logan *et al.* (1987), on the one hand that morulae grow with all *Cowdria* isolates and also with Jongejan *et al.* (1989), on the other, that *Cowdria* isolates did not all grow equally well in neutrophil cultures, some do not grow at all, but in this study they failed to grow at all only in some individual animals. Perhaps the isolates used by the authors should be tested further in new animals. From the evidence in this study, even low virulence isolates can achieve >1% culture infection rate making neutrophil derived antigen easy to produce from low virulence isolates. However 3 or 4 animals might need to be infected to increase the chances of obtaining one with a suitably high neutrophil infection rate. It is possible that Awa *et al.* (1995), by chance used stocks that were virulent thus more infectious to neutrophils or were tissue culture adapted and therefore problems of extremely low or no infection rate did not arise.

Three categories of Kenyan isolates emerged due to neutrophil cultures; prolific ones, non prolific ones and the in between. The Kiswani isolate could be regarded as special case in that it was derived from culture adapted endothelial culture elementary bodies, perhaps being more adapted to culture media. The more prolific isolates were more often also the more pathogenic in the sheep, and the least prolific the lowest in virulence.

4.4.4 ENDOTHELIAL CULTURES

While endothelial cell culture has been shown to be a far superior source of *Cowdria* (Bezuidenhout, 1987b), many factors affect the successful cultivation of *Cowdria ruminantium* (Bezuidenhout, 1987b). The organisms were first successfully cultured by (Bezuidehout *et al.*, 1985). The usefulness of these culture was the fundamental principle behind the effort put in this endeavour during these studies. By using plasma from animals with high and prolonged fever, Byrom *et al.* (1991), achieved a high success rate isolating from 85% of the animals with 73% positive cultures. The art of

growing *Cowdria* in endothelial cells was more daunting than anticipated supporting the findings of Yunker (1995). Most of the initial attempts failed. It was notable that eventually only the most virulent isolates were established in culture in our laboratories and from relatively few animals (Table 4.7). (The Asembo and Bamba infections were attempted from equally large number of sheep infected simultaneously with the Suswa, Kathiani 972 and Baragoi). (By contrast, neutrophils achieved a greater success rates with all isolates albeit most were at a very low level). The Isiolo, considered to be of intermediate virulence in this study, was established in Harare when a sheep there was inoculated with the blood stabilate of this isolate donated from the NVRC. Of the five cultures established by Mrs Smith, they took longer (13 to 20 days), than the average of 11 to 14 days found by Byrom *et al.* (1991. This would appear to support the observation that milder isolates are more difficult to establish than virulent ones. Perhaps they have a unique cell tropism other than endothelial cells as does the Kumm isolate (Du Plessis and Kumm 1971).

The total number of Kenyan heartwater isolates established in bovine pulmonary artery endothelial cells (BPA593), rose from one, the Kiswani, Kocan *et al.* (1987b), which had been established by Byrom *et al.* (1991), to five to include the Suswa, Baragoi, Kathiani 972 and Isiolo.

4.4.5 SEROLOGY AND WESTERN BLOTTING

The major immunogenic polypeptides (21, 32, 40, 46, 58, 85 and 160 kDa), of *Cowdria ruminantium* have been identified by western blotting (immunoblotting), Rossouw *et al.*, 1990) (immunoblotting and immunoprecipitation), Mahan *et al.* (1994b). There is strong evidence that the immunodominant antigens of *E. canis* and *C. ruminantium* contain cross-reacting epitopes, Kelly *et al.* (1994). Some of the pre-infection sera with levels closer to the cut off level in the cELISA detected and recognised few antigens in the Western blots in agreement with Kelly *et al.* (1994) and Jongejan *et al.* (1993a), that animals exposed to *Ehrlichia* cross react with *C. ruminantium* antigen showing in the 32 kDa and few other antigens on the Western blots. The high antibody titres in the recovery sera (Appendices 4.6a - c), was reflected in the numerous and strong bands in the Western blots (Figure 4.3a, b, c, &

d), seen with all isolates. The reactivity of antibodies in recovery sera embrace a number of antigenic epitopes which *Cowdria* shares with the *Ehrlichia* (Jongejan *et al.*, 1993a). Different sheep infected with the same isolate recognised more similar but not the same complement of immunogenic antigens. Jongejan (1991b), found that sera of goats infected with the Senegal isolate recognised the same pattern of antigenic bands in the western blots, albeit there was a change in the strength of recognition over time. Some antigens were more immunodominant in some sheep infected with different isolates and sometimes when the same isolate infected different sheep. These differences could have arisen due to differences in the isolates, treatment of some sheep as opposed to natural recovery (Section 4.3.7), or factors in individual animals affecting the response of the sheep to a particular antigen. The western blots were thus taken not to distinguish the different isolates, but as a strong indicator that the organisms isolated were *Cowdria,* and very close relatives of *Ehrlichia* (Jongejan *et al.*, 1993b).

4.4.6 AMPLIFICATION OF *COWDRIA* DNA FROM BLOOD EXTRACTS

The use of the PCR to detect infection in arthropods has been reviewed by Higgins, and Azad (1995), and DNA and oligonucleotide probes have been developed for *Cowdria* (Mahan, 1995; Peter *et al.*, 1995). A PCR based assay had been found highly sensitive and specific for detecting rickettsial and other tick borne diseases (Saiki *et al.*, 1988; Kock *et al.*, 1992; de Kok *et al.*, 1993). In PCR, a targeted segment of DNA is specifically synthesised by replication *in vitro* through a repeated cycle of three steps (Mullis and Faloona, 1987; Mullis *et al.*, 1986; Saiki *et al.*, 1985). The PCR performed in this case was *Cowdria* specific. Since PCR does not produce different reaction fragments, it could not be used to distinguish isolates of the same species, but it confirmed that the genome was present in the organisms/samples tested. e.g. Deem *et al.* (1996a), used a PCR based assay to detect the presence of *Cowdria* in ticks. The amplification of a product having a size of 388 bp in the isolates (Figures 4.2a-d), indicates that the agents in the isolates were very closely related to each other. The samples were however not all positive from the same animal or animals infected with the same isolate. The later could reflect on success of DNA

extraction or less than optimal conditions during the PCR reaction (Innis and Gelfand, 1990; Watson, 1993). There was amplification of more than one product for some of the isolates (Figure 4.2c), showing that there could have been a mispriming early in the amplification cycle resulting in two competing products, one being non-specific. The reduced ability to be amplified in some of the samples could indicate possible differences in the DNA base sequence of the 16S rDNA, without necessarily altering the amino acid sequence in the different *Cowdria.* To support this theory, these isolates fell into different groups when the GroE operon was tested based on PCR and RFLP analysis (Nicoll *et al.*, 1997 in preparation). Moreover, nucleotide sequence of MAP-1 genes from *Cowdria ruminantium* isolates from different geographical areas, have been found to differ by a base or two between isolates Reddy *et al.* (1996), who found sequence to differ from each other, by 0.6 to 14.0%. Although unlikely, the two different sizes in the doublet found in this study, may have arisen by acquisition or lose of a few bases thus giving a different size product from the regular one. The more likely reason is purely mispriming early in the amplification cycle but a product of correct size was also produced but was only weakly detected. The few samples amplified from all isolates may mean that the amplification and especially the annealing conditions were too stringent (too high for stability of the primer-amplicon hybridisation complex), for some of the isolates. This experiment has not differentiated individual Kenyan isolates but has separated them into two broad groups: those whose DNA was readily amplified and those that were less readily amplified by the HE 1(cr)/HE3(l) primers. Interestingly more samples of the more virulent isolates were positive (even in the same animal), than for the milder isolates. There is mounting evidence that just as there is a spectrum of antigenicity and immunogenicity among agents regarded as *Cowdria,* they also exhibit a spectrum of molecular inheritance, (Barbet *et al.* 1994; Reddy *et al.* 1996; Nioll *et al.* 1997). It would therefore seem appropriate that criteria used to differentiate *Cowdria* and *Ehrlichia* should include phenotypic and molecular characteristics.

4.5 Conclusion

Kenyan heartwater isolates were very similar to other *Cowdria* from South Africa, Zimbabwe, West Africa and the Caribbean in most respects: in spectrum of infectivity, incubation, virulence and post mortem pathology. This also included their detectability in the capillary endothelium of the brain, neutrophil cultures and also in the detection serologically, western blots and PCR.

There was difference in the fatality rate between the virulent isolates (those that killed all untreated animals), and the 'mild' isolates (those that spared some animals), as a group (Table 4.2a), which was extremely significant on the Fishers exact test ($p < 0.0001$). The diagnostic index of brain crush smears was positively correlated; high with virulent isolates and lower in the less virulent ones (Spearmans' correlation coefficient (r_s) = 0.957, $p < 0.0001$, considered extremely significant), meaning that most heartwater cases should be relatively easy to diagnose as most isolates were the virulent ones. But it also means that the milder ones are the ones that most likely become underdiagnosed. It is not possible to make a confirmatory diagnosis on all heartwater cases by brain crush smears, the mild isolates were again irresolute on this characteristic. The neutrophil infection rates are higher and more frequent with the more virulent isolates while infection rate in neutrophils is less frequent, in fewer sheep and usually reach a lower highest infection rate with the milder isolates. Some isolates may have a high neutrophil infection rate but a low diagnostic index, e.g. the Suswa isolate. Post mortem lesions may lack the suggestive hydrothorax and hydropericardium simultaneously and cause a confusion over the diagnosis, but the endothelium of brain capillaries may still have many colonies to confirm heartwater.

Table 4.1 Plan of isolates, stabilates, and the sheep used to determine the characteristics of Kenyan *Cowdria* strains in Corriedale sheep

Isolate	Stabilate no.	Day made*	Passage	Experiment/procedure	No of animals	Animal nos.
Asembo	352*	1	0	stabilate testing	2	62*, 63
				cross-immunity controls	6	20, 84, 81, 91, 140, 171
				tick infectivity	2	176, 177
Bamba	304	2	0	stabilate preparation	1	34**
	348	2	1	stabilate testing	2	55, 57*,
				cross-immunity controls	6	23, 97, 83, 102, 120, 129
				tick infectivity	2	183, 187
Baragoi	349	2	0	stabilate testing	3	40, 44, 59*
				cross-immunity controls	6	66, 100, 127, 166, 131, 161
				cross-immunity non-control	1	133
Galana 233	294	2	0	stabilate testing	4	238, 239*, 30, 31
Galana 229	305b	-*	0	stabilate testing	2	51, 61*
Isiolo	325	1	0	stabilate testing	2	38*, 39
Kathiani 32	330b	1	0	stabilate testing	2	35, 58
Kathiani 972	327b	2	0	stabilate testing	2	43, 46*
				cross-immunity controls	4	98, 103, 152, 155

Table 4.1 (continued)

Isolate	Stabilate no.	Day made*	Passage	Experiment/procedure	No of animals	Animal nos.
Kiswani	331	1	e*	stabilate testing	2	28, 36,
Marigat	311	2	0	stabilate preparation	1	48**
	345	2	1	stabilate testing	2	42, 47
Suswa	341	-	0	stabilate testing	2	53, 54*
				cross-immunity controls	6	15, 92, 116, 124, 119, 169
				cross-immunity non control	12	122, 125, 134, 136, 145, 147, 151, 156, 164, 165, 174, 175

Key

The design of this study has favoured stabilates that were used for more experiments.

Column 2 352* - this stabilate was made on the first day of fever from sheep 52 (it was feared that it would die like its predecessor, sheep 50 which died before a blood stabilate had been made)

Column 3 stabilates were made on the second day after temperature had reached 41°C,
-* means this animal reached less than this temperature (≥40.5°C).

Column 4 e* - early passage but unknown; tissue culture EBs used to infect sheep no. 33 to make the blood stabilate 331.

Column 7 cross immunity animal numbers put in the pair order in which they were used e.g. 20 & 84, 81 & 91, 140 & 171 showing that pairs were infected at chronologically different times

Column 7 * - animal treated with OTC on second day of fever ≥ 40.5°C to obtain serum

Column 7 ** - animals infected with respective primary blood stabilates to produce first passage stabilates; Bamba, St. 348 and Marigat, St. 345.

Column 7 81, 91 - underlined are homologous challenge animals infected with two millilitres of 1/3 equivalent blood stabilate.

Table 4.2a: Infectivity testing for stabilate of Kenyan *Cowdria* isolates: temperature and vital profiles for one millilitre of blood stabilate

Column	2	3	4	5	6	7	8	9	10	11	12
Isolate	Sheep #	Method of *Cowdria* diagnosis	Incubation	Days of fever	Max. temp. °C	Day to 40°C	41°C	recovery	Death	Total dead (%)	Reaction index
Suswa	53	a b	8	4	41.1	8	9	-	11		48.72
Suswa	15	a b	9	2	41.6	-	9	-	10		52.02
Suswa	92	a b	8	3	42.0	-	8	-	10		51.73
Suswa	119	a b	7	6	41.9	7	10	-	12		45.82
Suswa	169	a	10	1	41.2	-	10	-	10		52.73
Suswa	median	a b	8	3	41.6	7.5	9	na	10	5(100)	51.73
Baragoi	40	a b	11	3	41.3	-	11	-	13		51.77
Baragoi	44	a b	11	3(1)*	41.1	-	11	-	14		49.84
Baragoi	100	a b	9	5	42.0	9	10	-	13		49.42
Baragoi	66	a b	8	6	41.9	8	9	-	13		50.06
Baragoi	131	a	7	4(2)**	41.3	7	10	-	12		50.21
Baragoi	161	a b	9	5	41.9	-	9	-	13		51.25
Baragoi	Median	a b	9	4.5	41.6	8	10	na	13	6(100)	50.14
Kat.. 972	43	a b	10	4	41.2	-	10	-	13		52.54
Kat.. 972	98	a b	10	5	42.0	-	10	-	14		50.30
Kat.. 972	103	a b	9	5	41.6	9	10	-	13		47.35
Kat.. 972	152	a	10	4	41.9	10	12	-	13		49.56
Kat.. 972	155	a	10	4	41.7	10	13	-	13		48.42
Kat.. 972	median	a b	10	4	41.7	10	10	na	13	5(100)	49.56

Table 4.2a (continued)

Column	2	3	4	5	6	7	8	9	10	11	12
Isolate	Sheep #	Method of *Cowdria* diagnosis	Incubation	Days of fever	Max. temp. °C	Day to 40°C	41°C	recovery	Death	Total dead (%)	Reaction index
Kiswani	36	a b	10	5	40.8	10	-	-	14		43.15
Kiswani	28	a b	12	2	41.0	12	13	-	13		50.56
Kiswani	median	a b	11	3.5	40.9	11	na	na	13.5	2(100)	46.86
Gal. 229	30	a b	12	3	41.1	12	14	-	14		48.96
Gal. 229	31	a b	15	7	41.8	15	20	-	21		43.48
Gal. 229	median	a b	13.5	5	41.45	13.5	17	na	17.5	2(100)	46.22
Gal. 233	51	a b	12	3	40.7	12	-	-	15	1(100)	48.82
Isiolo	39	a b	12	7	41.8	12	13	-	20	1(100)	47.84
Marigat	42	a b	13	4	41.6	13	15	-	16		49.80
Marigat	47	b c	14	6	42.0	14	17	20	-		14.72
Marigat	22	a	14	5*	42.0	14	18	-	20		47.70
Marigat	median	b	14	4.5(na)	42	13.5(na)	16.5(na)	na	18	2(66.67)	47.70
Asembo	63	b c	9	9(1)	41.8	9	12	16	-		12.30
Asembo	20	b c	11	15	41.8	11	12	26	-		21.75
Asembo	84	a b	9	7	42.0	9	12	-	15		45.00
Asembo	176	a	7	6	41.9	7*	17	-	22		45.60
Asembo	177	c	16	7	41.9	-	16	23	-		14.52
Asembo	171	b	10	6	41.9	10	12	-	15		48.56
Asembo	140	a	12	3	41.6	-	12	-	14		50.40
Asembo	median	a (c)	10	6(9)*	41.9	9(10)	12(12)	23	15	4(57.14)	45.00

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Table 4.2a (continued)

Column	2	3	4	5	6	7	8	9	10	11	12
Isolate	Sheep #	Method of *Cowdria* diagnosis	Incubation	Days of fever	Max. temp. °C	Day to 40°C	41°C	recovery	Death	Total dead (%)	Reaction index
Bamba	55	b c	15	7	41.9	15	16	22	-		12.87
Bamba	23	b c	16	9	41.6	16	17	25	-		18.03
Bamba	97	b c	19	7	41.5	19	22	26	-		13.24
Bamba	183	c	18	7(1)	41.3	-	18	26	-		12.14
Bamba	187	c	14	9(2)	41.7	14	15	26	-		16.90
Bamba	120	c	17	3	40.3	17	-	20			2.30
Bamba	129	b c	14	8	41.6	14	16	-	21		44.14
Bamba	102	b c	17	7	41.8	17	18	24	-		11.76
Bamba	median	b c	16	7	41.6	16	17	25	na	1(12.50)	13.06
Kat.. 32	35	b c	13	6	41.6	13	15	19	-		11.04
Kat.. 32	58	b c	13	4	41.7	-	13	17	-		7.48
Kat.. 32	median	b c	13	5	41.65	na	14	18		0(0.00)	9.26
Overall/	median	a b	11	5	41.7	12	13	13	23	29(100)*	47.84

KEY

column 3 a - denotes confirmation by brain crush smears
b - denotes diagnosis by morulae in neutrophil cultures
c- diagnosis by seroconversion

Column 5 number in brackets means there was small peak(s) before the main one given
* temperature of this animal had a dip back to normal for one day in between.
** temperature of this animal had a dip back to normal for two days in between.

Column 11 - 29(100)* = the total number of deaths, 29 (out of 42) with a median mortality of 100% between isolates

Underlined - median values; for Asembo median died (recovered)**; na - not applicable.

Table 4.2b: Sheep inoculated with different volumes of blood and stabilate of Kenyan *cowdria* isolates: temperature and vital profiles

Isolate	Sheep #	Method of *Cowdria* diagnosis	Incubation	Days of fever	Max. temp. °C	Day to 40°C	Day to 41°C	Day to recovery	Day to death	Total dead (%)	Reaction index
Suswa+	**54**	**b c**	**9**	**3**	**41.6**	**9**	**11**	**12**	-		**4.97**
Suswa	116*	a	9	4	41.4	9	10	-	12		50.42
Suswa	124*	a	9	2	41.1	9	10	-	10	2	51.48
Baragoi	**59**	**b c**	**8**	**5**	**41.2**	**8**	**9**	**13**	-		**8.50**
Baragoi	127*	a b	9	7	41.7	9	10	-	15		45.64
Baragoi	166*	a b	10	5	41.7	10	11	-	14	2	50.70
Kat.. 972	**46**	**b c**	**10**	**6**	**41.6**	-	**10**	**16**	-	**0**	**11.58**
Kiswani	**33****	**b c**	**7**	**6**	**41.5**	**7**	**9**	**13**	-	**0**	**7.80**
Gal. 229	**239*****	**c**	**10**	**6**	**41.0**	**10**	**12**	**16**	-		**nd**
Gal. 229	238***	a	18	2	41.3	18	18	-	20	1	52.12
Gal. 233	**61**	**b c**	**16**	**4**	**41.7**	-	**16**	**20**	-	**0**	**8.50**
Isiolo	**38**	**c**	**13**	**6**	**41.7**	-	**13**	**19**	-	**0**	**12.32**
Asembo	**62**	**b c**	**12**	**7**	**41.8**	**12**	**13**	**18**	-		**11.30**
Asembo	81*	a	12	3	41.6	-	12	-	15		50.94
Asembo	91*	a	12	3	42.0		12	-	14	2	50.73
Marigat	48****	b c	12	6	41.6	12	13	18	-	0	8.78
Bamba	**57**	**c**	**16**	**4**	**41.6**	**16**	**17**	**20**	-		**6.66**
Bamba	34****	b c	9	9	41.4	9	15	24	-		13.66
Bamba	83*	b c	17	11	41.9	-	17	28	-	0	17.25

KEY: Data in this table not included in infectivity statistics; column 2 - * - denotes 1/3 ml stabilate equivalent diluted to 1 ml with RPMI 1640 medium used to infect this animal. (Other animals infected with one ml neat stabilate except as indicated below). ** - denotes this animal infected with tissue culture elementary bodies to obtain blood stabilate used. *** - denotes this animal infected with a large volume of blood stabilate. **** - denotes this animal infected with primary isolate to obtain first passage blood stabilate used. [+]Data in bold - of treated sheep that supplied sera for doing serology and western blots.

Table 4.3a: Number of control sheep (untreated) showing clinical signs during the course of infection with *Cowdria* blood stabilate

Isolate	No. of sheep in group	No. showing dullness	Rapid breathing	No. grinding teeth	No. lying down	No. showing incoordination	No. having diarrhoea	No. of deaths in group
Suswa	6	3	4	1	2	0	1	6
Baragoi	6	3	5	0	3	0	0	6
Kat. 972	4	4	4	0	0	0	0	4
Asembo	6	4	3	0	3	0	0	4
Bamba	6	1	2	0	3	1	0	1

Table 4.3b: Clinical manifestation of heartwater in sheep infected with *Cowdria* organisms derived from ticks (isolation animals)

Isolate	Sheep no.	Source	Incubation	Max. temp °C	Dullness	Rapid breathing	Grunting	Grinding teeth	Lying down	Prostration	ICD	Diarrhoea	Death	Reaction index (RI)
Suswa	965	nn	18♣	42	+	+	-	-	-	-	-	++	19	52.4
Suswa	41	guts	12♣	40.7	+	+	-	+	+	+	+	+++	14*	49.2
Baragoi	49	guts	7	41.7	+	+	+	-	+	+	+	-	11*	46.7
Kat. 972	972	guts	11	41.3	+	+	-	+	-	+	+	-	13*	50.4
Gal. 233	233	nn	16	41.1	-	-	-	-	-	-	-	-	17	51.7
Gal. 229	229	nn	20	40.7	+	+	-	-	+	+	+	-	22*	51.9
Asembo	50	guts	12♣	40.6	-	-	-	-	-	-	-	-	13	49.9
Asembo	52	guts	13♣	41.1	+	-	+	-	+	-	++	-	13*	52.8
Marigat	245	guts	16♣	41.8	-	-	-	-	-	-	-	-	18*	50.8
Marigat	29	guts	14	41.6	+	+	-	+	+	-	-	-	19*	47.3
Isiolo	27	guts	12	41.5	+/-	+	-	-	-	-	-	-	13*	51.5
Bamba	243	aa	13	40.4	-	-	-	-	-	-	-	-	21	48.8
Kat. 32	32	guts	11	41.4	+	+	-	-	-	-	+/-	-	18*	39.1

KEY:

column 1 - Gal. 229 = Galana 229 isolate, Gal. 233 = Galana 233, Kat. 32 = Kathiani 32, Kat. 972 = Kathiani 972

column 3 - nn = *Amblyomma* nymphs, guts = ground up tick supernatant, aa = *Amblyomma* adults

column 4 - ♣ only the definitive temperature rise shown here, some marginal or Dipen® responsive fever occurred before this

column 6-13 - -, +, +/-, ++, +++, = lesion absent, present, and degree of as perceived at examination

column 12 - ICD = in co-ordination

column 14 - * animal euthanased in *extremis*, otherwise animal found dead,

Table 4.3c: Clinical signs of sheep that died a per acute death before and inspite of treatment with oxytetracycline

Isolate	Sheep no.	Treatment	Sheep showing this sign: Dullness	Rapid breathing	Grinding teeth	Lying down	Lack of coordination	Diarrhoea	Day to death	RI
Suswa	122	N	-	-	-	-	-	(+)	10	48.3
Suswa	125	Y	-	-	-	-	-	+	10	53.1
Suswa	134	Y	-	+	-	-	-	(+)	10	51.7
Suswa	136	N	-	-	-	-	-	(+)	9	53.5
Suswa	145	N	-	-	-	-	-	(+)	10	50.3
Suswa	147	Y	-	-	-	-	-	-	10	51.5
Suswa	151	Y	-	-	-	-	-	-	10	51.2
Suswa	156	N	-	-	-	-	-	-	9	51.6
Suswa	164	Y	-	-	-	-	-	-	10	50.5
Suswa	165	Y	+	+	-	-	-	+	10	51.8
Suswa	174	Y	-	-	+	-	-	-	10	49.3
Suswa	175	N	-	-	-	-	-	(+)	9	51.1
Baragoi	133	Y	+	+	+	-	+	-	12	49.1

KEY

Y = yes; N = no; (+) = lesion only seen after death

Table 4.4a: Diagnostic index: number of sheep that died with different levels of colonies in the endothelium of brain capillaries: primary infection alone

Isolate	Level 1	Level 2	Level 3	Level 4	Total # sheep	Total points	DI
Kat. 972	-	-	1	5	6	55	91.7
Baragoi	-	-	4	6	10	80	80.0
Isiolo	-	-	1	1	2	15	75.0
Galana 229	-	-	1	-	1	5	50.0
Asembo	-	4	4	1	9	38	42.2
Suswa	2	9	7	2	20	73	36.5
Galana 233	-	1	1	-	2	7	35.0
Kiswani	-	2	1	-	3	9	30.0
Marigat	1	2	-	-	3	4	13.3
Bamba	2	-	-	-	2	0	0.0
Kat. 32	1	-	-	-	1	0	0.0

KEY

Diagnostic index (DI)) - score per animal x10 (Section 4.2.10)

Level	*Percent Colonies Seen*	*Score*	*Comment*
1	none	0	not possible/very difficult to diagnose in brain crush smear
2	< 1%	2	difficult to diagnose
3	1% - 4.9%	5	easy to diagnose
4	≥ 5%	10	very easy to diagnose

Table 4.4b: Diagnostic index: number of sheep that died with different levels of colonies in the endothelium of brain capillaries: primary and challenge infections

Isolate	Level 1	Level 2	Level 3	Level 4	Total # sheep	Total points	DI
Kat. 972	-	2	3	11	16	129	80.6
Baragoi	-	-	8	12	20	160	80.0
Isiolo	-	-	1	1	2	15	75.0
Galana 229 -	-	-	1	-	1	5	50.0
Kiswani	-	2	1	1	4	19	47.5
Asembo	-	4	5	1	10	43	43.0
Suswa	2	17	9	3	31	109	35.2
Galana 233	-	1	1	-	2	7	35.0
Marigat	1	2	-	-	3	4	13.3
Bamba	2	-	-	-	2	0	0.0
Kat. 32	1	-	-	-	1	0	0.0

KEY

Diagnostic index (DI) - score per animal x10 (Section 4.2.10)

Level	*Percent Colonies Seen*	*Score*	*Comment*
1	none	0	not possible/very difficult to diagnose in brain crush smear
2	< 1%	2	difficult to diagnose
3	1% - 4.9%	5	easy to diagnose
4	≥ 5%	10	very easy to diagnose

Table 4.5: Post mortem lesions in sheep that died of cowdriosis through out the study: the number and percentage of sheep having a lesions

Isolate	Sheep no. in the group	Colonies seen in brain capillaries	Nasal & subdural congestion	Lung lesions	Hydro thorax	H/ pericar-dium	Heart lesions	Full g/ bladder	Liver lesions	Kidney lesions	Spleen lesions	Ascites	GIT h'ges	Perineal soiling	Eye lesions
Suswa	20	18	5	19	15	10	20	20	19	20	14	6	4	10	1
{Suswa}	11	11	5	11	7	8	8	10	11	11	6	4	2	7	0
Baragoi	9	9	4	8	8	6	7	9	6	8	6	2	6	1	2
{Baragoi}	10	10	2	10	10	9	9	9	10	10	9	6	1	0	0
Kat. 972	6	6	0	4	5	6	3	5	6	6	4	3	1	0	0
{Kat. 972}	10	10	2	10	8	8	10	10	9	10	7	4	0	0	0
Kiswani**	3	3	0	3	2	3	0	2	2	2	1	0	3	0	0
{Kiswani}	1	1	0	1	0	1	1	1	1	1	0	0	0	1	1
Gal. 229	3	3	1	2	1	2	2	2	2	1	2	0	2	0	1
Gal. 233	1	1	0	1	1	0	0	1	1	1	0	0	0	0	0
Asembo**	6	6	1	5	5	5	6	6	4	6	3	2	0	0	0
{Asembo}	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Marigat	3	2	0	3	3	2	0	3	2	2	1	1	1	0	0
Isiolo	2	2	0	1	2	1	0	1	1	1	0	0	0	0	0
Bamba	2	0	1	2	2	1	2	1	2	2	1	1	1	0	2
Kat. 32	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0
Total	89(100)	83(93.3)	22(24.7)	81(91.0)	71(79.8)	64(71.9)	69(77.5)	81(91.0)	77(86.5)	83(93.3)	55(61.8)	29(32.6)	21(23.6)	19(21.4)	7(7.9)

KEY to Table 4.5

Kat. 972 = Kathiani 972; Kat. 32 = Kathiani 32; Gal. 229 = Galana 229; Gal. 233 = Galana 233; GIT = gastro-intestinal tract; h'ges = haemorrhages

{} - immune sheep given heterologous challenge with this isolate; ** mouse organ homogenates used to infect one of the animals

Brain colonies - sheep whose brain smears had no colonies were confirmed by morulae in the neutrophil cultures. The Marigat sheep did not have morulae either but it had the very enlarged gall bladder, lung lesions hydrothorax and hydropericardium.

Table 4.6: Development of morulae in neutrophil cultures of different isolates.

Cowdria Isolate	No. of Sheep	Febrile days	No of days cultures set up	Days culture +ve = x/y (%)	# days > 1%	Highest percent +ve (median)	RI of most highly infected sheep in the group	Note
Asembo	4	18	11	9/11 (81.8)	4/11	30(8)	52.8	H
Kiswani	4	14	7*	6/7 (85.7)	3/7	25(4)	50.6	H
Baragoi	4	18	13	7/13 (53.8)	2/13	3(<1)	46.7	I
Suswa	3	9	8	7/8 (87)	1/8	1(<1)	49.2	I
Bamba	3	20	18	5/18 (27.8)	2/18	1(1)	6.7	I
Kat.. 972	3	17	14	9/14 (64)	1/14	1(<1)	11.6*	I
Marigat	4	22	23	2/22 (9.1)	1/22	1(<1)	49.8	L
Kathiani 32	3	17	15	3/15 (20)	1/15	1(<1)	7.5	L
Isiolo	3	16	9	2/9 (22.2)	0/9	<1(<1)	47.8	L
Galana 229	2	10	6	1/6 (16.7)	0/6	<1(<1)	43.5	L
Galana 233	2	7	5	4/5 (80)	0/5	<1(<1)	48.8	L

KEY

Column 5 x/y is the proportion of cultures in which morulae grew out of the number that were prepared

Column 6 # days > 1% - the proportion of cultures that became infected with morulae in a minimum of one percent of neutrophils.

Column 7 indicates the highest percentage of neutrophils that were infected in any individual culture that was set up for the group of sheep infected with the isolate.

Column 8 * indicates the RI of this animal was low because it was treated. The median RI for this isolate was 49.6.

Column 9 H = high, I = intermediate, L = low, neutrophil infection rate.

Table 4.7: Isolation of *Cowdria* in endothelial cell cultures from blood of infected sheep.

Isolate	Number of sheep sampled	Number of sheep positive	Positive sheep numbers	Date culture isolated	Days taken to see EBs
Asembo	27	0	-	-	-
Bamba	27	0	-	-	-
Baragoi	27	4	66	13.1.96	20
			100	14.1.96	19
			127	18.4.96	24
			161	6.5.96	45
Galana 233	3	0	-	-	-
Galana 229	3	0	-	-	-
Kathiani 972	6	3	98	13.1.96	20
			103	13.1.96	34
			155	6.5.96	24
Kathiani 32	3	0	-	-	-
Isiolo	4	1	3-51	22.8.95	13-20
Marigat	4	0	-	-	-
Suswa	27	2	163	3.3.96	49
			116	14.4.96	25

KEY

- = not applicable

Table 4.8: Source of *Cowdria* DNA samples extracted for amplification using the HE1(Cr)/HE3 (l) primers

Isolate	Sheep sampled febrile blood	Mice sampled pleural fluid	Blood/fluid +ve	Neutrophil culture +ve	TC EBs control
Baragoi	40, 44, 49, 59	nd	40	40, 44, 49	na
Kiswani	28, 33, 36, 45	na	33	28, 36, 45	+ve
Kat. 972	43, 46, 972, 34	na	972	43, 46	na
Suswa	41, 53, 54	na	41	41, 54	na
Asembo	50, 52, 62, 63	c1dm4, c1dm5, c2dm6,	62, 63	-	na
Kat. 32	32, 35, 58	na	35, 58	35	na
Bamba	55, 57,34	na	34, 55	-	na
Isiolo	27, 38, 39	na	39	39	na
Marigat	29, 42, 47, 48	na	47	48	na
Galana 229	30, 31	na		31	na
Galana 233	51, 61	na	51	-	na
Ball 3	na	na	-	-	+ve
Gardel	na	na	-	-	+ve
Welgevonden	na	na	-	-	+ve

KEY

HE1(cr)/HE3(l) primers - primers that amplify a 388 bp on the 16S rRNA (DNA) gene of *Cowdria*

Table 4.9: The approximate western blot size (Kb) profiles obtained with immune sera to Kenyan isolates against *C. ruminantium* (Welgevonden) antigen.

Isolate	As	As	Bam	Bam	Bam	Bar.	Gal 29	Gal233	Isiolo	Kis.	Kat.32	Kat.37	Kat.972	Mar.	Suswa
serum	5935	5936	5582	4771	5583	6129	–	6131	4309		4772	5584	5205	4773	5416
d.p.i ~	28	28	28	28	28	28	40	28	28	28	28	28	28	28	28
treated	y	n	n	n	y	y	y	y	y	y	n	n	y	n	y
gel-track	5.2	11.3	5.3	11.4	13.4	5.4	3.2	7.2	7.4	3.3	9.3	13.2	9.2	9.4	11.2
quality	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
sheep	62	63	55	34	57	59	239	61	38	33	35	58	46	47	54
70-90	+	+	-	+	-	+	-	+	+	+	-	+	-	-	+
58	+	+	+++	++	+	+++	+	+	+	+	++	+++	++	++	+
40	+	+	+	+	-	+	+	+	+	+	+	++	+	+	++
32	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
29	+++	++	++	+	++	+++	+	++	++	+	-	++	-	+	++
27	+	+	-	-	++	+	-	-	-	+	-	-	-	+	+
24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+
Titre	4	16	16	128	nd	16	4	4	32	2	16	32	64	64	16

KEY

Column 1 - size of recognised protein in kDa

As - Asembo; Bam. – Bamba; Bar – Baragoi; Mar – Marigat; Kat. – Kathiani; Kis. – Kiswani; Gal – Galana; dpi - days post infection

+ weak positive; +++ strong positive; - negative; titre -reciprocal of end point; n – no; y - yes

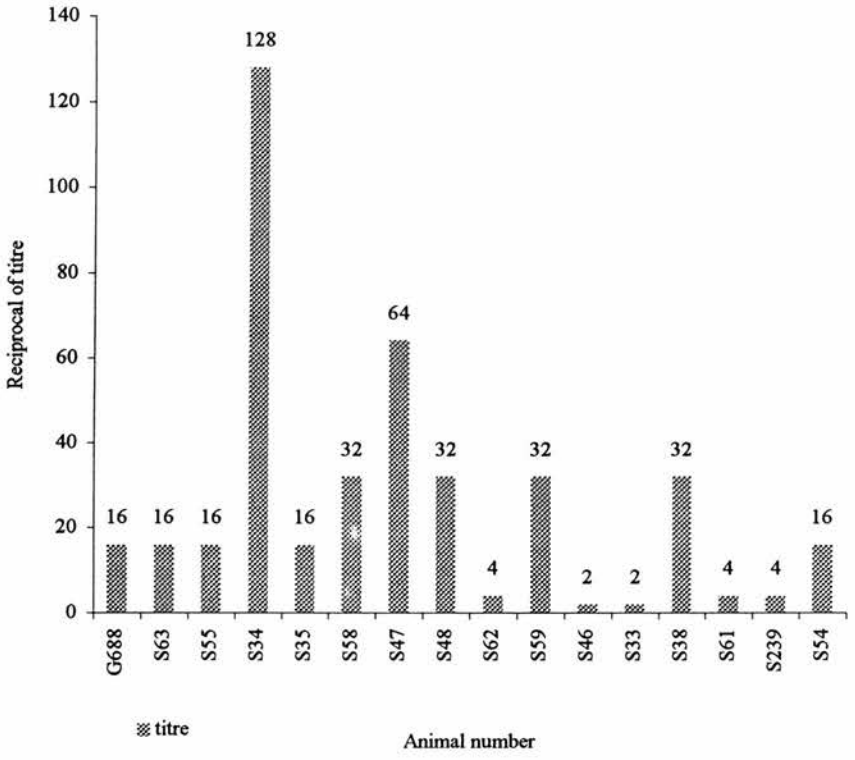


Figure 4.1 The reciprocal of titre by cELISA test of post infection sera obtained from heartwater infected sheep used to perform western blots

KEY

Anti-serum	An. no.	Titre	Note	Anti-serum	An. no	Titre	Note
Asembo	S63	16	nat. rec.	Baragoi	S59	32	treated
Bamba	S55	16	nat. rec.	Kathiani 972	S46	2	treated
Bamba	S34	128	nat. rec.	Kiswani	S33	2	treated
Kathiani 32	S35	16	nat. rec.	Isiolo	S38	32	treated
Kathiani 32	S58	32	nat. rec.	Galana 233	S61	4	treated
Marigat	S47	64	nat. rec.	Galana 229	S239	4	treated
Marigat	S48	32	nat. rec.	Suswa	S54	16	treated
Asembo	S62	4	treated				

nat. rec. - natural recovery
An. no. - Animal number
S63 - sheep number 63
G688 - goat number 688
Cut off level for the titration = 50%
Positive control serum - homlogous for *C. ruminantium* (Welgevomden) antigen used (G688 source)

Key (figure 4.2a)

LANES	SAMPLE	SHEEP	RESULT	LANE	SAMPLE	SHEEP	RESULT
1	Ball 3 tc (udg.)	na	-	16	Gardel tc	na	+
2	Welgevonden tc	na	+	17	Bamba	34(n)	-
3	Gardel tc	na	-	18	Suswa	41(n)	-
4	Kiswani tc	na	+	19	Marigat	29(b)	-
5	Galana 229	31(n)	+	20	Marigat	29(b)	-
6	Isiolo	39	+	21	Marigat	29(b)	-
7	Galana 229	31(b)	-	M	MWt	na	-
8	Kathiani 972	972(b)	+	23	Galana 229	30(b)	-
9	Isiolo	27(b)	-	24	Galana 229	30(b)	-
10	Suswa	41(b)	+	25	Isiolo	39(b)	+
M	MWt	na	na	26	Isiolo	38(b	+
12	uninfected	49(b)	-	27	Kathiani 32	32(b)	-
13	Rgts blank	na	-	28	Kiswani	33(b)	-
14	Welgevonden tc (udg.)	na	-	29	uninfected	49(b)	-
15	Ball 3 tc	na	+	30	Rgt blank	na	-

tc - EBs positive control DNA from tissue culture

tc EBs digested unless indicated as (udg.)

Mwt - DNA size standards (BioRad)

Rgts blank - reagent only

uninfected = negative control

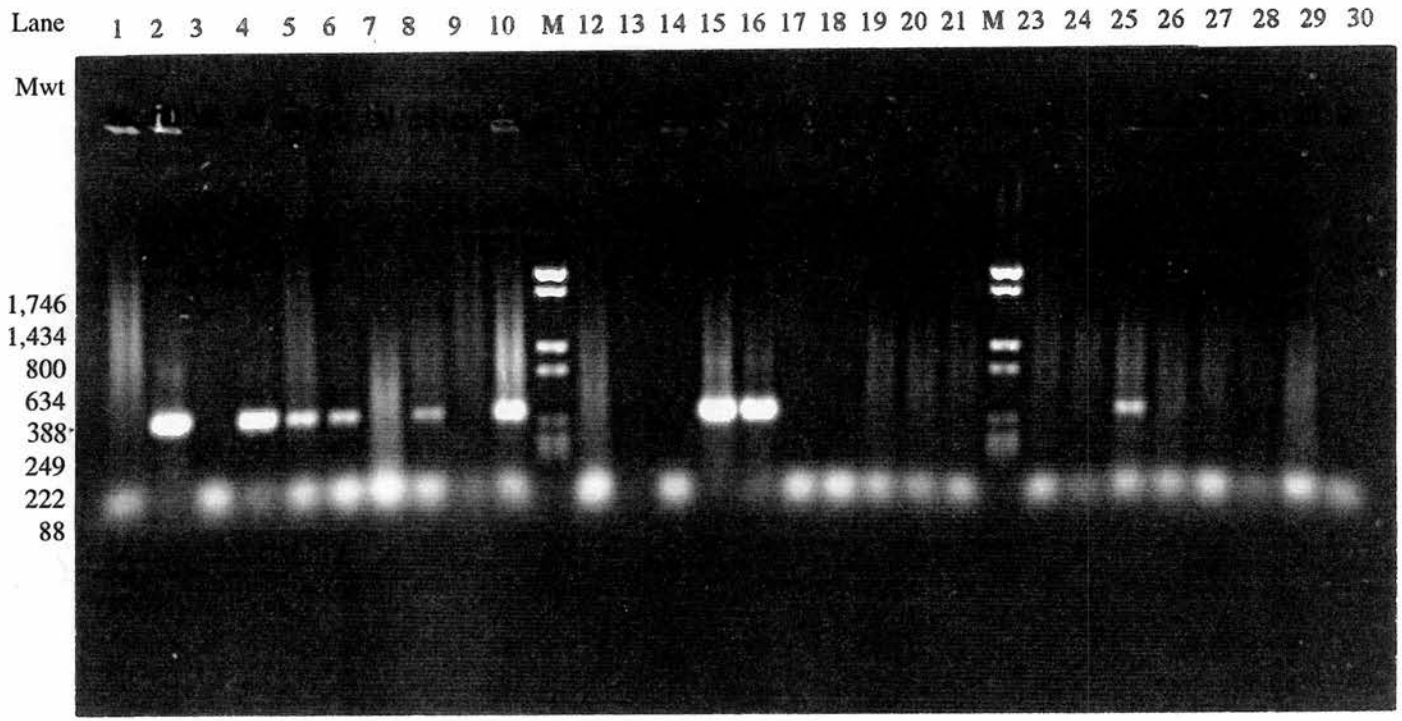


Figure 4.2a: Amplification of *Cowdria* DNA extracts from blood and neutrophil cultures using the HE1(Cr)/HE3 (1) primers

Key (figure 4.2b)

LANES	SAMPLE	SHEEP	RESULT	LANE	SAMPLE	SHEEP	RESULT
1	uninfected	59(b)	-	16	Marigat	48(n)	-
2	Welgevonden tc	na	+	17	Marigat	48(n)	-
3	Rgt blank	na	-	18	Bamba	34(n)	-
4	Kiswani	45(n)	-	19	Bamba	34(n)	-
5	Kiswani	45(n)	+	20	Kiswani tc	na	+
6	Baragoi	49(b)	-	21	uninfected	60(b)	-
7	Baragoi	49(b)	-	22	Bamba	34(n)	-
8	Baragoi	49(b)	+	23	Bamba	34(n)	-
9	Baragoi	49(n)	-	24	Bamba	34(n)	-
M	MWt	na	-	25	Bamba	34(n)	-
11	Baragoi	49(n)	+	26	Bamba	34(n)	-
12	Baragoi	49(n)	-	27	Bamba	34(n)	-
13	Baragoi	49(n)	+	28	Asembo	52(b)	-
14	Marigat	48(n)	-	29	Marigat	42(n)	-
15	Marigat	48(b)	-	30	Baragoi	44(n)	+

tc - EBs positive control DNA from tissue culture

uninfected = negative control

Mwt - DNA size standards (BioRad)

Rgt blank - reagent only

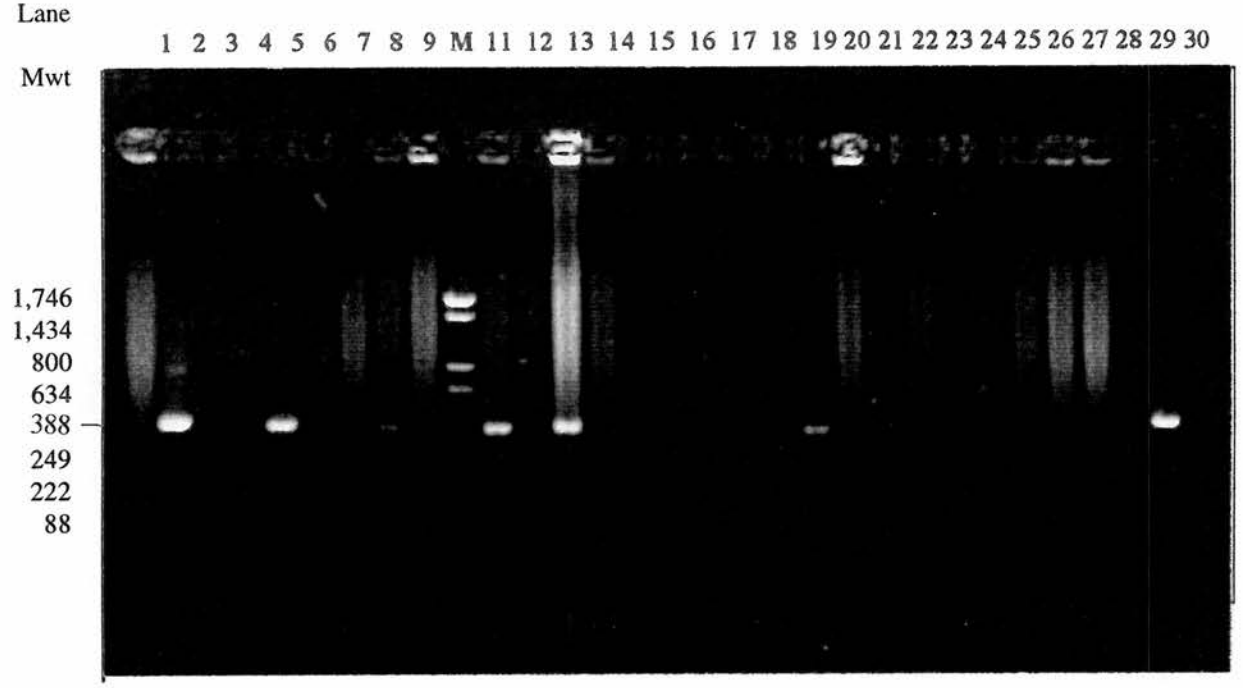


Figure 4.2b: Amplification of *Cowdria* DNA extracts from blood and neutrophil cultures using the HE1(Cr)/HE3 (1) primers

Key (figure 4.2c)

LANES	SAMPLE	SHEEP	RESULT	LANE	SAMPLE	SHEEP	RESULT
1	uninfected	59(b)	-	16	Bamba	34(b)	-
2	Welgevonden tc	na	+	17	Bamba	34(b)	-
3	Rgt blank	na	-	18	Bamba	34(b)	+
4	Marigat	47(b)	+	19	Bamba	34(b)	+
5	Marigat	47(b)	-	20	Welgevonden tc	na	+
6	Marigat	47(b)	+	21	uninfected	62(b)	-
7	Marigat	47(b)	-	22	Bamba	34(b)	+
8	Marigat	47(b)	-	23	Kathiani 32	35(b)	-
9	Marigat	42(n))	-	24	Kathiani 32	35(b)	+
M	MWt	na	na	25	Marigat	42(b)	+
11	Marigat	42(n)	-	26	Marigat	48(n)	+
12	Marigat	42(b)	+	27	Marigat	48(n)	-
13	Marigat	42(b)	-	28	Marigat	48(b)	-
14	Marigat	42(b)	-	29	Marigat	48(b)	-
15	Bamba	34(b)	+	30	Marigat	48(b)	-

tc - EBs positive control DNA from tissue culture

uninfected = negative control

Mwt - DNA size standards (BioRad)

Rgt blank - reagent only

* - notice the different size product in these lanes and doublets in lanes 14 & 19.

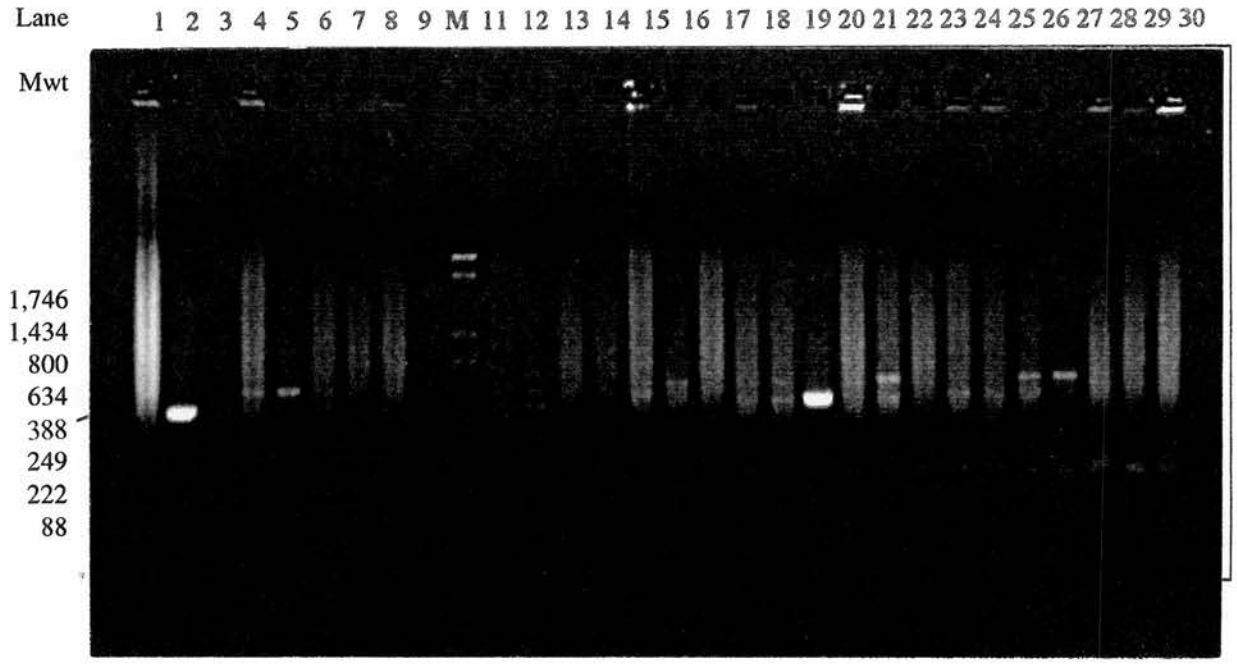


Figure 4.2c: Amplification of *Cowdria* DNA extracts from blood and neutrophil cultures using the HE1(Cr)/HE3 (1) primers

Key (figure 4.2d)

LANES	SAMPLE	SHEEP	RESULT	LANE	SAMPLE	SHEEP	RESULT
1	uninfected	56(b)	-	16	Asembo	62(b)	+*
2	Welgevonden tc	na	+	17	Asembo	62(b)	+*
3	Rgt blank	na	-	18	Kathiani 32	58(b)	-
4	Kathiani 972	46(b)	-	19	Kathiani 32	58(b)	+*
5	Suswa	54(n)	-	20	Welgevonden tc	na	+
6	Marigat	47(n)	-	21	uninfected	55(b)	-
7	Bamba	34(b)	-	22	Kathiani 32	58(b)	-
8	Kathiani 32	35(b)	+	23	Kathiani 32	58(b)	+
9	Kathiani 32	35(n))	-	24	Kathiani 32	58(b)	-
M	MWt	na	na	25	Kathiani 32	58(b)	+
11	Kathiani 32	35(b)	-	26	Kathiani 32	58(b)	-
12	Asembo	62(b)	-	27	Kathiani 32	58(b)	+
13	Asembo	62(b)	-	28	Kathiani 32	58(b)	-
14	Asembo	62(b)	-	29	Kathiani 32	58(b)	+
15	Asembo	62(b)	-	30	Asembo	63(b)	+

tc - EBs positive control DNA from tissue culture

uninfected = negative control

Mwt - DNA size standards (BioRad)

Rgt blank - reagent only

+* - notice the different size product in these lanes (doublets)

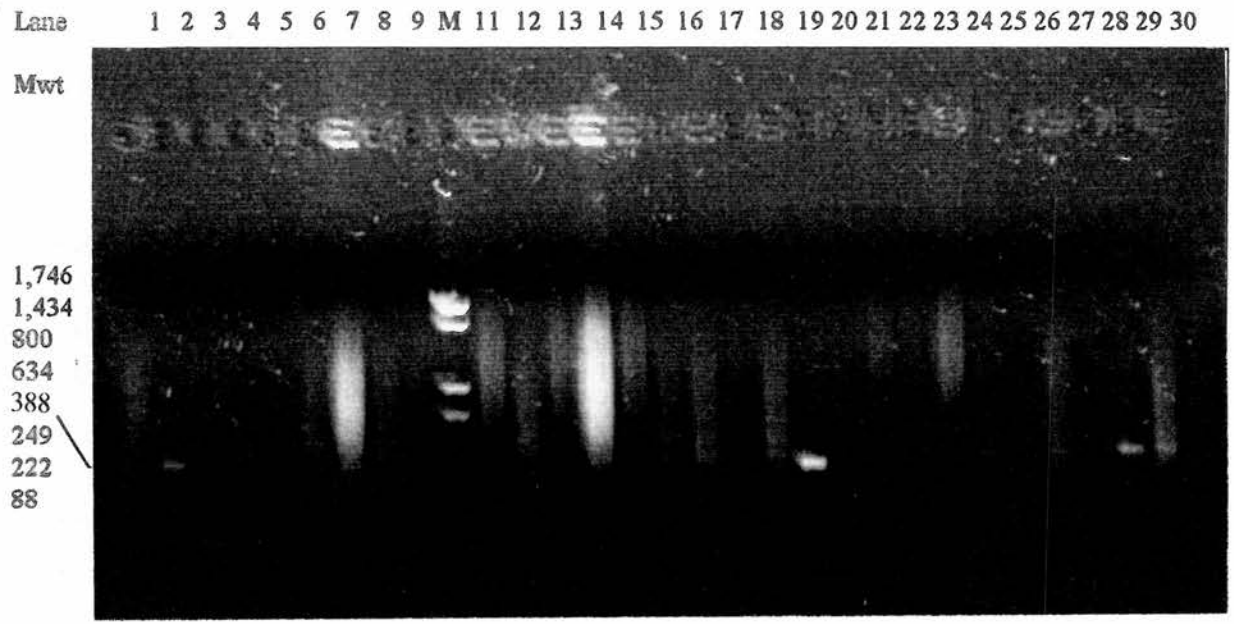
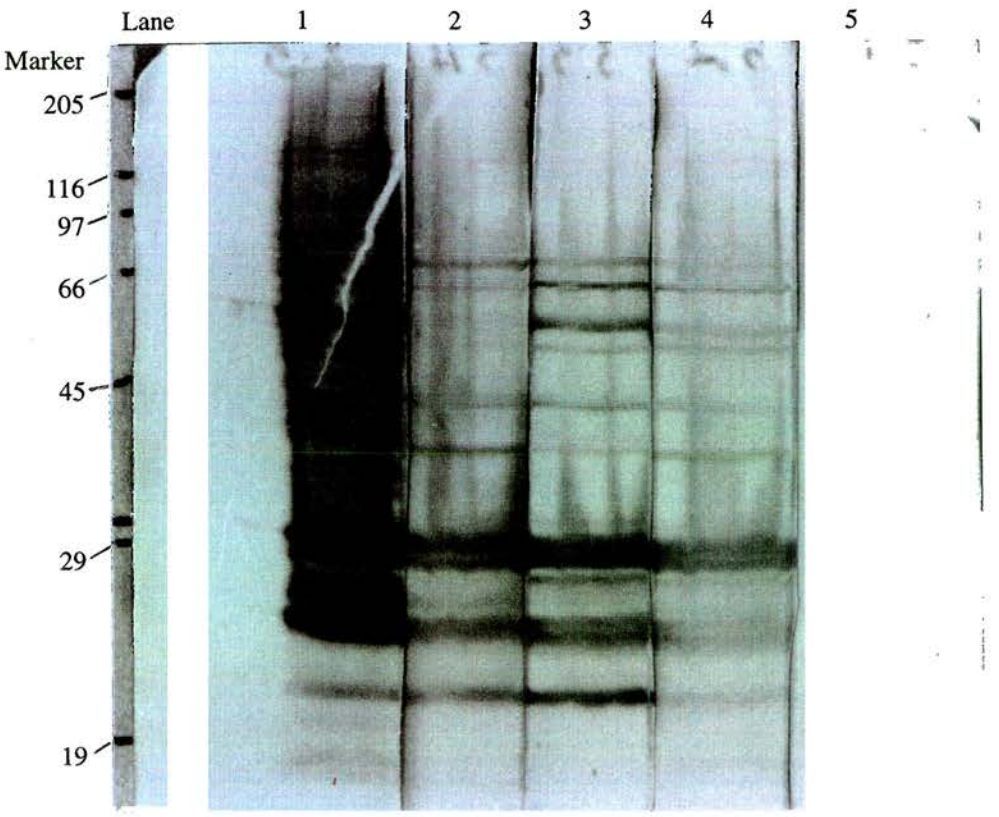


Figure 4.2d: Amplification of *Cowdria* DNA extracts from blood and neutrophil cultures using the HE1(Cr)/HE3 (1) primers

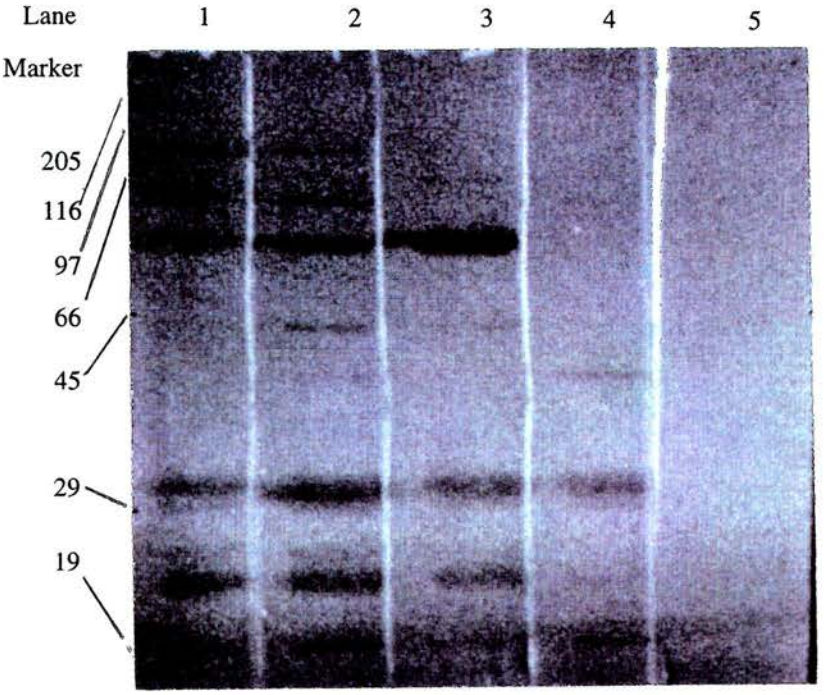


KEY

Lane	Serum	Animal #
1	Welgevonden	G668
2	Gal. 233	239
3	Kiswani	33
4	Marigat	963
5	uninfected	29

Mwt - High molecular weight marker

Figure 4.3a: SDS-PAGE western blot of sera from sheep infected with different *Cowdria* isolates

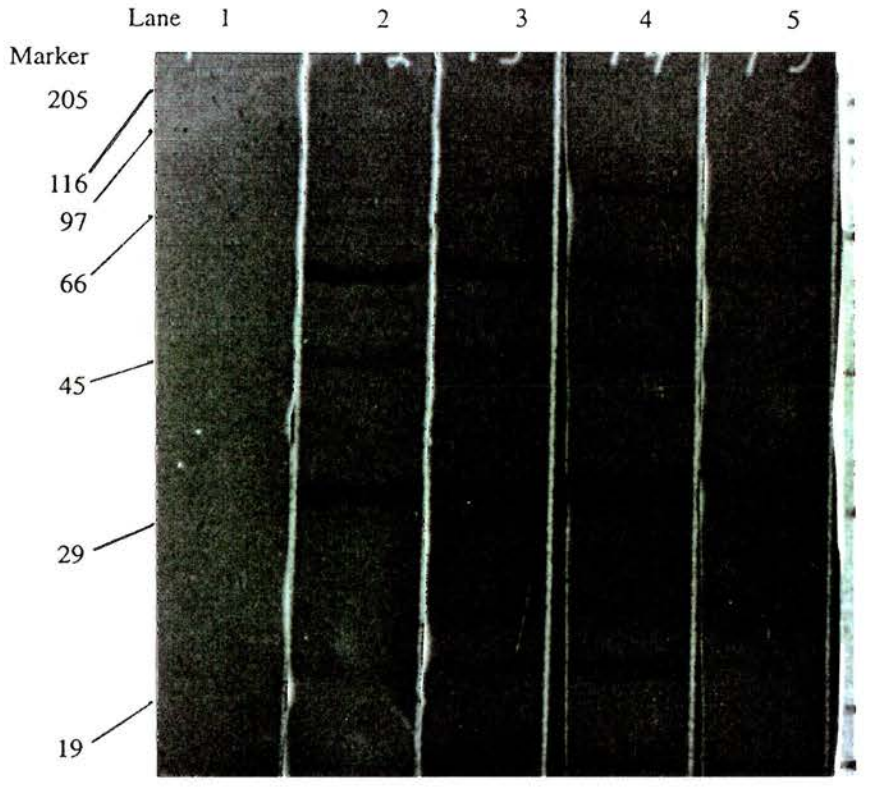


KEY

Lane	Serum	Animal #
1	Welgevonden	G668
2	Baragoi	59
3	Bamba	55
4	Asembo	62
5	uninfected	63

Mwt - High molecular weight marker

Figure 4.3b: SDS-PAGE western blot of sera from sheep infected with different *Cowdria* isolates

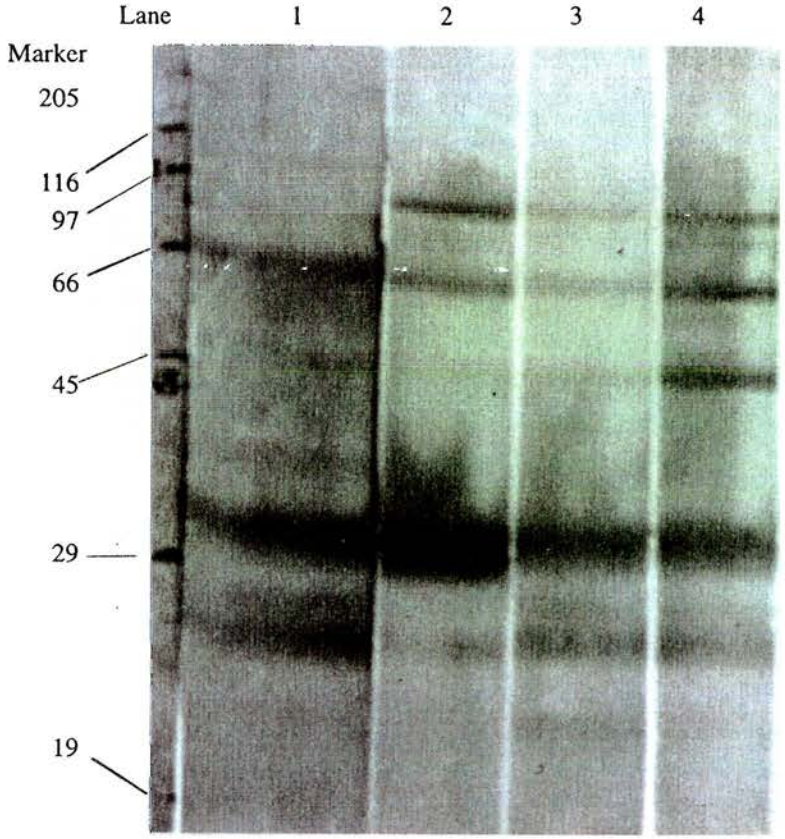


KEY

Lane	Serum	Animal #
1	uninfected	63
2	Marigat	47
3	Kat.. 32	35
4	Kat.. 972	46
5	Welgevonden	G668

Mwt - High molecular weight marker

Figure 4.3c: SDS-PAGE western blot of sera from sheep infected with different *Cowdria* isolates



KEY

Lane	Serum	Animal #
1	Welgevonden	G668
2	Suswa	54
3	Asembo	63
4	Bamba	34
5	uninfected	63

Mwt - High molecular weight marker

Figure 4.3d: SDS-PAGE western blot of sera from sheep infected with different *Cowdria* isolates

(a)

(b)

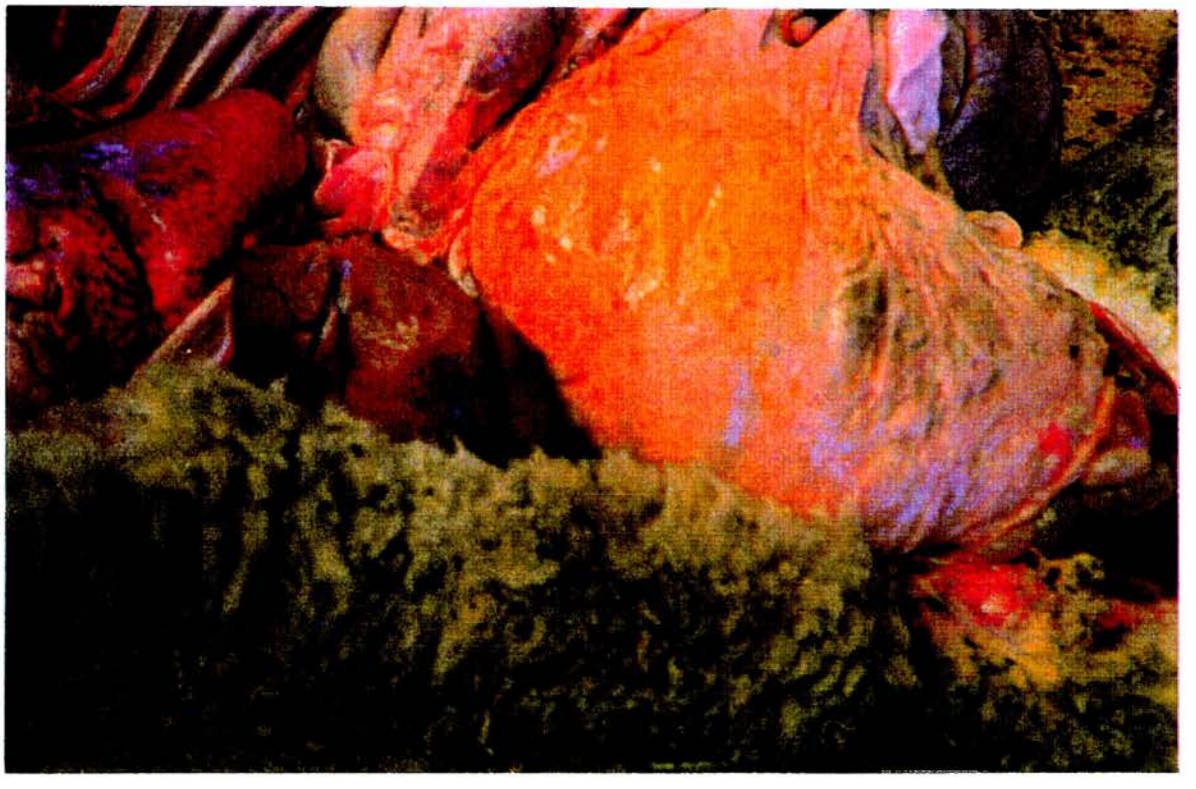


Figure 4.4 (a) A very full gall bladder; (b) bile staining of omental fat.

CHAPTER FIVE

CROSS IMMUNITY TRIALS IN SHEEP USING *COWDRIA RUMINANTIUM* ISOLATES FROM DIFFERENT GEOGRAPHIC LOCATIONS

5.1 Introduction

Stringent tick control measures have been practised in Eastern and Southern Africa for most of the last century mostly to prevent the transmission of the protozoal disease East Coast fever (ECF), by the ixodid tick *Rhipicephalus appendiculatus,* to exotic cattle and their crosses. If the introduction of immunisation of cattle against ECF leads to control of the disease, farmers are likely to relax the amount of acaricide control and adopt a more strategic approach to tick control. This would inevitably permit tick burdens of all species of ticks to increase on livestock, and with them tick-borne diseases such as cowdriosis, babesiosis and anaplasmosis (Uilenberg, 1984). This will increase the demand for the introduction of immunisation against these tick-borne diseases to keep them under control and to speed up the adherent endemic stability. Finding a suitable vaccine against heartwater has been previously considered the most useful outcome of practical benefit to the farmer (Neitz and Alexander, 1945), and the same must still apply.

The 'infection and treatment' immunisation for heartwater was first developed by Neitz and Alexander (1945). Today, the same procedure is still followed and comprises the inoculation of virulent blood and subsequent treatment with oxytetracyclines of animals that become febrile as a result of this artificial infection (FAO, 1984). Heartwater frequently manifests as an acute disease, usually terminating in sudden death after onset of fever. It is necessary for animals to become infected in order to become immune but treatment of the animals must be early and be continued until the temperature subsides. Recently one attenuated strain protected against homologous lethal challenge (Jongejan, 1991b), and prospects for vaccine have dramatically changed with the reports that animals can be protected with inactivated preparations (Tafesse, 1992; Martinez *et al,. 1*993c, 1994; Mahan *et al.,* 1995). However the immunity of some animals may be compromised when animals are treated early (Du Plessis *et al., 1*983), and on the other hand only a minority of cattle react with fever on homologous or heterologous challenge as observed by several authors, Karrar (1960), Ilemobade (1976), Van Winkelhoff and Uilenberg (1981). Because of the effect of early oxytetracycline treatment, a second inoculation of *Cowdria* is recommended before heterologous challenge in cross immunity studies

(Uilenberg *et al.*, 1983). Uilenberg (1983) has reviewed the role played by humoral antibodies. The influence of antibody to the course of infection has been investigated in studies by Du Plessis, (1970a, 1993) and Byrom *et al.*, (1993). However, the serological response is valuable in evaluating heartwater immunisation (Lawrence *et al.*, 1993).

A vaccine has been used to good advantage in South Africa for a long time (Bezuidenhout, 1989). Certain breeds of cattle, sheep and goats are known to be more susceptible than others (Van Der Merwe, 1979). Exotic animals being exposed to *Cowdria ruminantium* for the first time, are fully susceptible (Matheron *et al.*, 1987). Cross-protection between stocks of *C. ruminantium* has been found to be incomplete (Brown *et al.*, 1989; Du Plessis and Van Gas, 1989; Stewart, 1989; Jongejan *et al.*, 1988; 1991b; Du Plessis *et al.*, 1990b) or absent altogether (Jongejan *et al.*, 1991b). Although the Ball 3 stock has been used as a vaccine in South Africa for many years, the Welgevonden stock conferred a much broader spectrum of protection against the other South African stocks than did other immunising strains (Du Plessis *et al.*, 1989). For this reason, it would seem logical to identify a stock inducing broad protection for immunisation before adopting any of the Kenyan isolates. In this study an attempt was made to determine a suitable vaccine from among eleven Kenyan isolates by doing a two way cross-immunity trial. Five isolates from different geographical locations were selected. The choice of isolates was principally on the basis of virulence, two lower virulence isolates were chosen to determine if these could protect against three high virulence strains. The strains also originated from widely dispersed locations and had been isolated from *A. variegatum* or *A. gemma* ticks (chapter 2) and differed in their infection of mice (Chapter 6). The outcome of the study was evaluated by the extent of cross-protection in the immunized groups. The extent of cross protection would also provide an indication to the risk of heartwater for animals moving into and between *Amblyomma* areas.

5.2 Materials and methods

5.2.1 EXPERIMENTAL DESIGN

A preliminary experiment was performed with the eleven Kenyan *Cowdria* isolates in a one way cross immunity to generate information that would help in the choice of

stabilates to choose for a two way cross-immunity trial. This information included high virulence against which protection is sought and low virulence which is a more acceptable characteristic in vaccine candidates. As shown below, five isolates viz. the Asembo, Bamba, Baragoi, Suswa, and Kathiani 972 isolates were chosen for the two way trial on the basis of virulence, tick source and geographic representation (Chapter 2) and used as shown in Table 5.1. The first four isolates were used for immunisation, booster challenge and in heterologous challenge. The fifth isolate, Kathiani 972, a virulent isolate was used to challenge a subgroup of sheep in each of the immunisation groups because the information on homologous protection was obtained during the booster infection for each isolate.

Virulence characteristics in sheep can be found in Chapters 4 and characteristics in mice found in Chapter 6. Briefly the five *Cowdria* isolates were: Asembo, a moderately virulent isolate for sheep derived from *A. variegatum*, prolific in the brain and neutrophil cultures, and pathogenic for Balb/C mice. The Bamba isolate, a mild isolate for sheep, derived from *A. gemma*, not prolific in the brain or neutrophil cultures and not pathogenic for mice. The Suswa isolate from *A. variegatum* which had a high virulence for sheep, not prolific in the brain endothelial cells, but moderately prolific in neutrophil cultures and not pathogenic to mice. The Baragoi isolate, derived from *A. gemma* ticks, virulent for sheep, prolific in the brain and neutrophil cultures, and pathogenic for Balb/C mice. The fifth strain, Kathiani 972, had been isolated from *A. variegatum*, virulent to sheep but not mice, prolific in the brain and neutrophil cultures. They represented a good cross section of stocks from across the country, different vectors and pathogenicity for sheep and mice.

5.2.2 SHEEP

Heartwater susceptible corriedale sheep as described in Section 2.2, negative to *Cowdria* antigen on the cELISA (Section 2.6.1) were used. They were kept in a large tick free barn and their temperature was recorded daily during the acclimatisation period. They were kept tick free for the duration of the trial (around three months) by rubbing a pour-on along the back and underside once per month.

5.2.3 IMMUNISATION

5.2.3.1 STABILATE HANDLING

The stabilate was quickly thawed from liquid N_2 after the sheep had been restrained. Four tubes were immersed into a water bath at a temperature of 37°C. The stabilate after thawing was decanted into a universal bottle which was then kept cool on crushed ice. The stabilate was mixed before use and when one lot had been used another was thawed and used in a similar manner. All stabilates were used within twenty minutes of thawing. The sheep were observed for a few minutes for possible anaphylactic reaction so as to be treated should this occur.

5.2.3.2 SHEEP INFECTION

ONE WAY TRIAL

These sheep were infected by intravenous (i/v) inoculation of blood stabilate and recovered either naturally or following treatment in other experiments (Chapter 4) and therefore not all had been treated with OTC (Table 5.2a & b).

TWO WAY TRIAL

Heartwater naive sheep were immunised by the 'infection and treatment' method (Neitz and Alexander, 1945) using blood stabilate. Eighty sheep were divided randomly using the Block randomising Method (Bancroft, 1968) (Section 2.2.1) into 4 immunisation groups of 20 sheep and each immunisation group divided into four challenge groups of five sheep . Two naive sheep used as challenge controls were also included for each challenge group but not during primary infection (Table 5.1).

Each sheep was inoculated with one millilitre of blood stabilate intravenously into the jugular vein. All sheep (20 total per immunisation group) were given a second inoculation of stabilate fourteen days after the last animal in the group had been treated. They were given 0.3 ml equivalent of homologous blood stabilate diluted in 2 ml of RPMI 1640 culture medium (GIBCO BRL, UK). Two control sheep were inoculated to demonstrate stabilate infectivity.

5.2.4 CHALLENGE

ONE WAY CROSS-IMMUNITY

Challenge stocks were selected and matched empirically as animals became available. All animals except two had unique immunisation and challenge stock combinations but, not all possible combinations were tried. Challenge stabilates had previously been shown to be viable and capable of inducing infection in naive animals.

TWO WAY CROSS-IMMUNITY

Heterologous challenge occurred 21 days after homologous boost, or 21 days after the last animal was expected to have reacted with the low virulence (Bamba) isolate. Five sheep were injected with 1 ml of undiluted heterologous blood stabilate with four groups for each immunising stabilate. Two naive sheep were positive controls for each challenge group.

5.2.5 MONITORING

ONE WAY TRIAL

Sheep in the one way trial were those that survived in the infection studies (Chapter 4) and were therefore monitored as described in Section 4.2.4.

TWO WAY TRIAL

After immunisation all sheep were monitored as described in Section 2.2.3. During 'infection and treatment' immunisation, animals were treated on the second day of temperature greater than 40.5°C with 20 mg/kg body weight with long acting oxytetracycline (Tetroxy LA, Bimeda, UK). A second dose was given 48 hrs later if fever had not subsided. Five sheep immunised with the Suswa stocks, died before they could be treated on the second day of fever $\geq$ 40.5°C (Table 4.3c).

No treatment was given for temperature reaction occurring after booster and heterologous challenge. Sheep were monitored daily by early morning rectal temperature and visual inspection for clinical signs as described above. Animals were allowed to run their course during heterologous challenge and euthanased *in extremis*.

5.2.6 EVALUATION OF REACTION TO INFECTION AND CHALLENGE

5.2.6.1 REACTION INDEX

The severity of the reactions to infection of each sheep was evaluated by calculating the reaction index (Du Plessis, 1985). This has been described in detail in Section 2.2.3.1. but briefly, a score was obtained for the severity of the febrile reaction and an additional score was given for fatality to an infection. The two scores were added together to give a reaction index (RI.). For example, a sheep might have a score of 7.6 to the febrile reaction and because it died on day five, have a fatality score of 39 giving a total of 46.6 (i.e. RI = 46.6). If this sheep had not died or was treated and recovered, it would have a reaction index of 7.6.

5.2.6.2 PROTECTION LEVEL

RI METHOD

The reaction indices for each sheep were calculated for the three phases (immunisation, booster and challenge) of the trial (Appendix 5.3). The mean of the RI of the group of sheep immunised and challenged with various isolates was tabulated (Table 5.3). The difference between the mean RI for the challenge group compared with the (unimmunised) controls was taken to be the protection factor, and the percentage it constituted the protection level.

SEVERITY SCORE METHOD

A reaction score was calculated by determining the severity of reaction (classified as none, mild, severe and very severe) in individual animals in a challenge group. Points were awarded differentially for each level and the mean score for the group was calculated (Tables 5.4 & 5.5). The severity of the reactions to challenge of the immunised group of sheep was the average score of the group compared with the mean score of the control sheep. The difference was taken to be the protection level for the immunising isolate against the challenge isolate.

5.2.7 SEROLOGY

Serum was collected pre-infection, on day 28 post immunisation and day 21-28 days post boost and heterologous challenge. These were submitted to the cELISA test as described in Section 2.6.1.

5.3 Results

The details of individual animal vital responses during each of the three infections (immunisation, boost and heterologous challenge) is given in Appendix 5.1. & 5.2

5.3.1 RESPONSE OF SHEEP TO PRIMARY IMMUNISATION INFECTION

ONE WAY TRIAL

Sheep recruited into the one way cross immunity trial were deemed to have become immune to the respective isolate after recovering from the disease episode, either by themselves or after treatment with oxytetracycline (Tetroxy LA, Bimeda, UK). This was supported by other parameters (Section 4.3.3, 4.3.5-4.3.7, Tables 4.9, 4.10, Appendices 4.3 & 4.6a, b, &c) and only the sheep showing evidence of infection were subsequently challenged.

TWO WAY TRIAL

In the two way cross immunity trial, sheep were treated on the second day of fever (40.5°C). It was possible to contain the infection in all twenty sheep infected with the Bamba and Asembo isolates using this procedure. Exceptionally one sheep (no. 2) infected with the Bamba isolate reached a temperature maximum of only 40.2°C but was treated with OTC as this stock had killed the isolation animal without provoking a high fever. One sheep (no. 7) also infected with the Bamba isolate was not treated as it did not register any temperature $\geq$ 40°C (Appendix 5.1).

It was not possible to contain the infection of all sheep infected with the Baragoi and Suswa isolates by treating sheep on the second day of fever (40.5°C). One Baragoi infected sheep died of nervous signs one day after treatment, while 12 sheep infected with the Suswa isolate died suddenly before and after treatment (Table 4.3c). Because of these losses, the immunising protocol was changed for the replacement sheep for both stabilates. Sheep were monitored twice a day, morning and evening, and treated at the first rise of fever (≥40°C) around the expected day, and watched so as not to be treated later than the mean day of incubation for that stabilate. Because of this, three Suswa infected sheep (132, 137 & 153) were treated after detection of fever in the evening. One of these sheep (132) subsequently registered morning fever on the following three days including evening fever, but fever was detected only in the

evening in the other two sheep.

5.3.2 RESPONSE OF SHEEP TO HOMOLOGOUS BOOST INFECTION

A summary of the animal reaction experienced by sheep in the different immunisation groups after re-inoculation of sheep with homologous isolate is shown in Tables 5.4 & 5.5 and for individual animals in Appendices 5.1 & 5.2.

There were no obvious clinical signs seen after the homologous boost and none of the immunised sheep died despite absence of treatment. The mean RI (Table 5.3), as well as reaction severity score (Table 5.4) differentiated the magnitude of reaction in the different immunisation groups. The mean severity of the group reaction (Table 5.4) was reduced by over 80% in all immunisation groups compared to the control sheep (Table 5.4). At the same time the mean RI was reduced by over 90% in all immunisation groups (Table 5.3 Appendix 5.3) compared to the control sheep. Each of the control sheep infected with Asembo, Baragoi and Suswa died, while both of the Bamba infected controls survived, one having reacted with a high fever and the other with none (Appendix 5.4).

5.3.3 HETEROLOGOUS CHALLENGE

Protection was considered total if an animal did not exhibit thermal and/or clinical signs. The protection was considered partial if hyperthermia and/or clinical signs were detected, and an animal was considered to be unprotected if it died as a result of infection. The degree of protection was determined more precisely by examining the reduction in reaction indices and severity score.

ONE WAY TRIAL

The out come of challenge of sheep in the one way cross immunity trial is summarised in Table 5.2a. There was a spectrum of protection ranging from full protection to no protection against the challenge isolates. Seven out off twenty animals showed no thermal or clinical reaction (NR) after challenge. Four sheep died, following challenge with Kathiani 972 (one sheep), Kiswani isolate (one sheep), and the Suswa isolate (two sheep). Nine sheep had various degrees of febrile reaction and recovered. There was a slight positive correlation between the reaction indices of these sheep between the primary infection (median RI for isolate in the uninfected sheep, from Table 4.2a)

and protection factor (r = 0.133 Pearson's correlation MiniTab) but it was considered not significant (Figure 5.1a). The median reduction in the reaction index fell on partially protected sheep, (RI = 73.5%, range negative (-7%) to 100%). This means that animals had exacerbated reaction (sooner deaths) at one end and sheep were fully protected at the other end (Figure 5.1a).

TWO WAY TRIAL

The two way cross immunity trial has been summarised in Table 5.5. and the individual animal reactions are summarised in Appendices 5.1 & 5.2. The severity of reaction ranged from no sheep protected to all sheep protected in the different immunisation and challenge combinations (Tables 5.5 & 5.6). The mean protection level offered by each isolate against the other isolates put together (in ascending order) was, Bamba (14.0%), Asembo (18.8%), Suswa (67.1%), and Baragoi (98.8%) (Table 5.6). At the same time, the reaction indices registered a marginal decrease with the milder isolates (1.4% and 9.5% for the Asembo immunes and 7.6% to 18.0% for the Bamba immunes) against virulent challenge (Table 5.3). There was moderate to considerable decrease in reaction indices with the virulent isolates (55.3 - 97.5% for the Suswa and 98.3 -100% for the Baragoi (Table 5.3)). The reduction in the RIs was reflected in the reduction in severity score (Tables 5.5 & 5.6). There was an upward shift of vital parameters observed (increased median and/ or range of incubation period, increased duration of fever and increased days to death) following immunisation with virulent stocks. In contrast, when sheep were immunised with the milder (Asembo and Bamba) isolates and challenged with the Suswa isolate, the median and /or range of these parameters were shifted downward (Table 5.8a, b, & c in bold). This also appeared to have happened to a lesser extent in the sheep immunised with Bamba and challenged with the Baragoi and Kathiani 972 isolates.

Only two sheep immunised with the milder isolates survived when challenged with the virulent isolates. The Bamba isolate protected one sheep against the Kathiani 972, while the Asembo isolate protected one sheep against Suswa (Tables 5.5). Both sheep had a rise in their incubation periods and duration of fever which is reflected in the upper range for these isolates (Tables 5.8a & b). Neither Asembo nor Bamba isolate

protected any sheep against the other two isolates.

None of the 40 sheep immunised with the virulent isolates died of heterologous challenge whether mild or virulent. (Table 5.5). Protected sheep whether against mild or virulent isolates had parameters with a shift toward chronicity with longer incubations, shorter durations of fever and reduced deaths (Tables 5.8a, b, & c). This was in turn reflected in their reduced reaction indices and increased score for the level of protection.

All *Cowdria* naive (control) sheep (4 per challenge group) challenged with the Suswa, Kathiani 972 and Baragoi died while three Asembo, and one Bamba died. This is reflected in their reaction indices (Appendices 5.3) and protection score (Table 5.3 & 5.6).

5.3.4 ASSESSMENT OF IMMUNISATION EFFICIENCY

Immunity was judged to have been conferred on the basis of the serological response seen in the majority of sheep (Appendices 5.1 & 5.2) and the ability to control the challenge of the homologous boost infection (Table 5.4). In the serological response measured by the cELISA test (Section 2.6) all the twenty Asembo infected sheep, 17 out of 20 Bamba, 17 out of 20 Baragoi, and 18 out of 20 Suswa infected sheep had increased cELISA IL that reached cut off level of 70% inhibition. After boosting only three sheep had not seroconverted (reached the 70 % cELISA inhibition level): two Bamba and one Suswa infected sheep. Only in the two Bamba infected sheep the antibody remained unaltered and these were considered to be non seroconverters.

The serological responses of sheep to immunisation with the four isolates (Appendices 5.1 & 5.2) was not identical but could be grouped into two basic patterns: those that had a significant inhibition level (IL) difference between infection and the boost infections and those that IL did not change significantly (Table 5.9, Appendix 5.5). The virulent isolates fell into the 'significant difference' category while the less virulent isolates fell into the 'non significant difference' category (Appendix 5.5). The majority of sheep infected with the Asembo isolate(75%) and Bamba isolate (70%), appeared to reach a maximum IL after the primary infection, and either remained in a plateaux or decreased at the end of the boost period (Appendix 5.1 & Table 5.6). On

the other hand 50% of Suswa infected sheep and 60% of Baragoi infected sheep reached only mild to moderate IL above the 70% cut off after primary infection (Appendix 5.2 & Table 5.6). Most IL continued to rise in Suswa (70% of sheep), and Baragoi (95% of sheep), to their maximum at the end of the boost period.

Percent cELISA inhibition patterns (Table 5.7), showed that there was increased IL after heterologous challenge in the majority of sheep that survived virulent challenge after being immunised with the milder strains. However, the majority of sheep immunised with the virulent isolates showed a decreasing IL, Suswa (50% of sheep), and Baragoi (65% of sheep), by the end of heterologous challenge. One Suswa infected sheep which had IL below the 70% cut off level remained at this level even after homologous and heterologous challenge, perhaps indicative of cellular immunity.

5.4 Discussion

At the end of this trial, there was strong support corroborating the observations of (Du Plessis, 1982), that in heartwater, immunogenicity is parallel to pathogenicity. The observations made in the one way cross immunity were strongly reinforced by those found in the two way cross immunity trial with selected isolates. If virulent isolates are usually the most protective, it would explain the finding that Welgevonden was the most broadly protective (as well as most virulent) (Du Plessis *et al.* 1989; Jongejan *et al.* 1991b). Ranking the amount of overall protection afforded by each immunising isolate against the other isolates in ascending order, placed the mildest isolate at the bottom and the second most virulent isolate to Corriedale sheep, at the top of the protectiveness scale.

It was after the Kumm stock was isolated (Du Plessis and Kumm, 1971), that the idea of differences in the different *Cowdria* isolates was circulated but only gradually accepted. Whereas Uilenberg *et al.* (1983), found few or no immunological differences, they have been well established in subsequent cross immunity trials (Jongejan *et al.*, 1991b; Jongejan *et al.*, 1988; Brown *et al.*, 1989; Du Plessis *et al.*, 1989), in which various degrees of cross-protection in sheep and goats has been established between local and exotic *Cowdria* stocks.

In the one way cross immunity study a range of reactions were observed. While some

sheep died as though they were heartwater naive or worse off, others showed no response to challenge (Table 5.2a & b). The increased, rather than decreased reaction indices, and the increased rapidity of disease development of Asembo, Bamba and Kathiani 32 immunised sheep challenged with the Suswa, Baragoi and Kathiani 972 isolates (Tables 5.2b, 5.8a, b, & c and Appendix 5.3), suggests that some of these sheep might have become more susceptible to the virulent isolates after recovering from the milder ones, (even more susceptible than naive sheep).

Sheep in the one way trial fell into three categories: those that were solidly protected (and had concurrent reduction in RI >90%); those that were partially protected (had concurrent reduction in RI in the 70 to 89 percent bracket); and those that were not protected also echoed by the magnitude of their reaction indices (reduction was very small, <10% or negative) (Table 5.2b). There were no immunisations that lead to protection in the 11 to 69% regression.

Du Plessis and Malan (1988), considered an RI score of 10 (in challenge cattle), to reflect a moderate febrile reaction as it was unaccompanied by other clinical signs. In sheep Du Plessis *et al.* (1989), considered a RI of 10 to be the cut off score above which the reaction of the infected animal was considerable. This is supported in my studies by a score of 13.21 (also representing a reduction of 73.3% in the RI), in the Bamba recovered sheep which when challenged with Kathiani 972 isolate in the one way studies survived (Table 5.2a), but four out of five sheep immunised with the Bamba isolate died of Kathiani 972 isolate challenge in the two way trial. This implies that the sheep in the one way trial escaped death narrowly. The animals in immunisation/ challenge combinations which had RI scores below 10 may not have been in any danger from the challenge isolates. It was interesting from the one way study that the virulent isolates (ones that had killed all untreated sheep, Table 5.2a), controlled the challenge against the other virulent isolates, but even more stimulating that the Marigat which failed to kill some naive sheep, protected against Baragoi challenge with a 98.5% reduction in RI.

The immune responses of cattle (and other ruminants), to *Cowdria ruminantium* are not well understood (Uilenberg, 1983; Stewart, 1987a; McKeever, 1993). *Cowdria*

are intracellular bacteria endowed with the capacity to invade, survive and replicate primarily in endothelial and neutrophils, but also other cells of their hosts. To do this, intracellular bacteria employ potent evasion mechanisms that their hosts normally employ to kill many bacterial invaders (Kaufmann, 1993). In these cross immunity studies low virulence isolates protected against other low virulence isolates but did not protect against high virulence isolates. However the high virulence isolates protected against high virulence isolates as well as the low virulence isolates. This would suggest that the immunity induced by the two 'types' of *Cowdria* isolates was different in some fundamental way and it is immunity that was induced by the virulent isolates which endured against assault of virulent *Cowdria*. The difference in the type of immunity induced between the virulent and lesser virulent isolates is hinted at in the pattern of serological response. The difference in IL between the isolates is most marked between the post infection and post booster IL (Table 5.7, Appendices 5.1, 5.2, 5.5). The difference in IL post infection compared with post booster IL, was not significant in the Asembo or Bamba anti sera, but it was significant in the Suswa anti sera ($p<0.05$), and extremely significant in the Baragoi anti sera ($P< 0.001$). Other levels of significance between the IL of the other sera is seen between the post infection and post heterologous antisera (summarised in Table 5.9). These differences are comparable to increases in IFA titres found by Du Plessis *et al.* (1989) in immunised animals with certain (not all) heterologous challenge combinations. The regression of the RI of challenge isolate on the RI of the immunising isolate (Figure 5.1b), might be an indicator of the immunologic relationship between the immunising and challenging agents as recognised by the host: a dissimilarity stimulates antibody production while a similarity does not stimulates antibody.

It is well established that IgG1 isotype is formed in a primarily TH2 T cell mediated responses while TH1 cell mediated responses favours the production of IgG2a isotype antibody (Stevens *et al.*, 1988). Antigen presenting cells (APC), process and present microbial proteins as antigens into the class 1 or class II MHC pathway initiating T cell activation, and simultaneously cause a divergence on the ensuing T helper cell response (Kaufmann, 1993). This has been observed for *Cowdria* in an *in vitro* milieu (Bourdoulous *et al.*, 1993; 1995). Stimulated cytokine producing cells respond by

cytokine gene transcription (formation of gene products), which travel to the ribosomes for mRNA translation to synthesise a cytokine (Delannoy *et al., 1*993). The released soluble cytokine finds target responder cells via the circulatory system, initiating the process that leads to counter attack of the invader. T helper cell functions have been separated into two main groups according to the lymphokines that they synthesise after activation by APC: (1), those which synthesise interleukin 2 (IL-2), and interferon gamma (IFN-γ) are CD4 α/β TH1 cells according to Kaufmann, 1993), and (2), those which synthesise interleukin 4 (IL-4) are TH2 cells (Mosmann and Coffman, 1989). Mosmann and Coffman (1989), call a third class cytotoxic lymphocytes (CTL), while Kaufmann (1993), categorises the third class of T cells as cytotoxic CD8 T cells, both authors appear to be referring to the same cells (although no doubt, more subclasses exist). All three categories of cells also produce other cytokines. Both authors elaborate the crucial role of TH1 T cells in cell mediated immunity but (Mosmann and Coffman, 1989), also elaborate on the TH2 cells in their role in humoral and cell mediated immunity and pathogenesis. Delannoy *et* al (1993), classifies the cytokines (rather than the cells that produce them), according to their functional role in inflammation hence, pro-inflammatory, anti-inflammatory and regulatory cytokines, all being produced by more than one cell type. Cytokine producing cells include monocytes, macrophages, fibroblasts, neutrophils, T-lymphocytes endothelial cells and keratinocytes and chondrocytes (Delannoy *et al.*, 1993). The three authors help to clarify the immunological processes that may be taking place in cowdriosis.

Kaufmann, (1993) depicts the polymorphonuclear and mononuclear phagocytes as the professional phagocytes and also the major effectors of antibacterial defence. According to Kaufmann, (1993), CD4 α/β TH1, cells activate mononuclear phagocytes and convert them from bacterial habitat to potent effector cells. He elaborates that this occurs by inducing them to generate reactive oxygen intermediates (ROI), which are toxic for bacteria; reactive nitrogen intermediates (RNI), which stop the growth and multiplication of bacteria; limitation of intracellular iron availability which is crucial for bacterial survival; phagosome acidification and phagosome-lysosome fusion; and production of defensins which kill the bacteria. CD8 T cells

induce lysis of host cells allowing release and subsequent uptake by more efficient phagocytes.

Obligate intracellular pathogens like *Cowdria*, avoid antibody responses, in their intracellular niche, but processing and presentation of their proteins promote T cell stimulation (Kaufmann, 1993), triggering hostility toward them by the above mechanisms. Thus protection in cowdriosis has been predicted and been shown to be mediated by T cell immune mechanisms (Totté *et al.*, 1997; Du Plessis *et al.*, 1991, 1992b). Immunological studies on heartwater have also shown that antibodies may not play a protective role in immunity in sheep (Du Plessis, 1970; Martinez *et al.*, 1994), and in mice (Byrom *et al.*, 1993). Hyperimmune serum had no influence on the outcome of the disease (Alexander, 1931; Du Plessis, 1982; Ramisse, 1971 cited by Uilenberg, 1983). Calves born from immunised cows with demonstrable colostral antibodies were no more resistant to challenge than calves without antibody (Camus, 1987), while immune sheep with high titres of antibody were partially or fully susceptible to heterologous challenge (Du Plessis, 1982; Du Plessis and Van Gas, 1989; Du Plessis *et al.*, 1989). Moreover, no antibody was detectable with the IFA test in the serum of 42 cattle that were resistant to challenge (Du Plessis *et al.*, 1992b). The effect of reinfection upon antibody level was to decrease and to drive levels to negative status in some of the animals (Du Plessis and Malan, 1987b). This was also the case in this study in a good number of animals immunised with the virulent isolates.

The role of TH1 in normal immune responses is enhancement of cytotoxic mechanisms (via IFN-γ primarily), such as activating macrophages so as to increase killing of intracellular parasite, and lysis of target cells hiding or harbouring the parasites (Mosmann and Coffman, 1989). The role of TH2 cells (via IL-4 primarily), is to increase levels of antibody in general (and IgE specifically), and increase mucosal mast cell proliferation and eosinophils (largely allergic type reactions) (Mosmann and Coffman, 1989). The effectiveness and appropriateness of each type of immunity was clearly demonstrated by Scott *et al.* (1988), using mice infected with *Leishmania*. Many normal immune responses involve mixed TH1 and TH2 responses (Parish,

1972). It is known that the ratio of TH1 to TH2 cells that are stimulated in certain infections, is important to give rise to the appropriate protective immunity e.g. against helminths (Ogilvie and Jones, 1969; Kelly and Ogilvie, 1972).

It is possible that the antigens of the milder *Cowdria* isolates activate an immunological response that is largely TH2 T cell mediated and the virulent strains initiated a more TH1 T cell mediated response. Interferon gamma has been associated with immunity in cattle which affected *Cowdria* in culture (Totté *et al.*, 1994). The ratio of TH1 to TH2 cells produced via various immune responses appears to be tightly controlled as assessed by clones generated in tissue culture and by the characteristic responses elicited by particular antigens (Mosmann and Coffman, 1989). A subsequent encounter triggers a similar immunological response as in the first encounter. Although the antigen presenting cell (APC), may be responsible for influencing the TH1/TH2 ratio, the physical nature of antigen encountered might influence the TH1/TH2 ratio by providing different accessory signals (Janeway *et al.*, 1988). The later theory would fit in with the findings that sheep immunised with the virulent strains (their antigens induced a protective immunity), were protected against both milder and virulent isolates, whereas those immunised with the milder strains (their antigens did not induce protective immunity against virulence), were not protected against challenge by the virulent isolates but only to 'homologous' challenge with other mild isolates. It might even explain why some of the sheep immunised with the milder isolates appeared to be more susceptible to the virulent isolates than naive animals (Tables 5.8a, b, &c). Since recall responses are often similar to the first response made in the primary infection, (a feature called 'original antigenic sin'), these sheep probably responded to the challenge by producing IL-4.

Uptake of bacteria can occur via the Fc region after immunoglobulin binding, which induces ROI production (Kaufmann and Reddehase, 1989), and the bacteria are killed, but endocytosis occurs via complement receptors (CR), after deposition of C3b or other breakdown product of complement, without provoking ROI production and the bacteria are unharmed. Cell invasive bacteria express molecules called invasins which facilitate bacterial entry in a similar manner to complement (Isberg, 1991). A

possible theory of events that took place in sheep immunised with 'mild' *Cowdria* isolates, is that the virulent *Cowdria* readily infected the sheep. They multiplied unchecked by the 'wrong response'. The TH1/TH2 interegulatory mechanism (Parish, 1972), exerted a negative feedback (by producing anti IL-4 anti bodies and other regulatory compounds), which stopped the 'wrong response,' enhanced the production of 'protective' interleukin (IFN-γ), response (since some TH1 cells would have also been stimulated in the primary infection). By this time however, there may have been numerous rapidly multiplying organisms and IFN-γ caused them to be killed, and parasitised endothelial cells to be lysed. The presence of a high level of killed bacteria produces endotoxin which leads to endotoxic shock: endotoxins have been reported in fatal heartwater (Van Amstel *et al.,* 1988b, 1994). An allergic reaction is also highly likely due to overproduction of products in a TH2 type of response and certain deaths in cowdriosis are so sudden one is reminded of an anaphylactic shock.

Comparable data is not available to compare previous cross immunity trials. However it appears that the stocks used in many studies are all highly virulent in the systems used (Jongejan *et al.,* 1991b) and some originate across borders. The relationship between virulent *Cowdria* appears to be more complicated and no pattern in antigenic diversity could be derived from cross immunity studies (Du Plessis *et al.* 1989 & 1990b). However, virulence still appear to be relevant to cross protection to a degree: there was considerable loss in cross protection when the attenuated Senegal was used (Jongejan *et al.,* 1993b) compared to the unattenuated one (Jongejan *et al.,* 1988; 1991b) against the Ball 3 and Welgevonden isolates.

The isolates of high virulence in this study did not all equally protect against each other which could be due to a spectrum of differences between their protective antigens. Janeway *et al.,* (1988) suggested that TH1 cells can only be activated by high antigen density on the surface of the antigen presenting cells, perhaps differences in the density and/or other conformational properties of the antigen of the different isolates, might have induced slightly different ratios of TH1/TH2 responses. This could explain why there was lack of equal cross protection between any two isolates

in the two way cross immunity, as observed before (Jongejan *et al., 1*988, 1991b), and also explain why a combination of a number of stocks did not improve immunogenicity (Du Plessis *et al., 1*990b; Jongejan *et al., 1*991b). If what Janeway *et al.,* (1988) suggests is correct, then it means that suitable immunising antigens should also be assessed on the basis of their density. As Van Kleef *et al.*, (1993), found that the 31kDa antigen derived from the Welgevonden stock was not protective, it might mean that immunodominance (Jongejan and Thielemans, 1989) does not reflect immunoprotectiveness. Since only 8 antigens have been well defined (Mahan, 1995), it would appear that not all *Cowdria* antigens observed by Rossouw *et al.,* (1990) (20 maximum in the Welgevonden), have been defined and could offer a wider choice to select from. Also, Nicoll *et al.* (1997) constructed a phylogenic tree from the amino acid sequences of the GroE operon of various isolates which grouped *Cowdria* isolates regionally though not exclusively. The Welgevonden and Senegal isolates which do not well cross-protect were a good distance away from each other which could mean that although *Cowdria* are conventionally considered to be monophyletic, there might be significant differences in their relatedness (Reddy *et al,* 1996) to the extent that they do not cross protect at all against each other. A case in point is the Welgevonden isolate which failed to protect many animals against Mali and Comoro isolates (Du Plessis *et al.,* 1989) which originate from other regions. The host species are also usually not equally suceptible to any one isolate as seen in the sheep and goats outcome (Jongejan *et al.,* (1991b). In this study one isolate that was virulent to cattle, sheep and mice was also the most protective in Corriedale sheep against the other four isolates. It might be that virulence to more host species increases cross-protection.

Immunogenicity of antigens can be improved by use of a suitable adjuvant which predisposes the immune response against the accompanying antigens to go in the same direction as the adjuvant response (Mosmann and Coffman, 1989). This has in fact already been done with Freunds or oil based adjuvants and the *Cowdria* EBs antigens and found to induce protective immunity (Mahan *et al.,* 1995; Martinez *et al., 1*994; Totté *et al.,* 1997). An interleukin IL-12 has also been found to have good adjuvant potential (Mahon *et al.*, 1996; M. Mutunga, pers. com.). However, if the

observations of Janeway *et al.,* (1988), are valid, then the author deduces that the immunity facilitated by this procedure might approximate that which the isolate would confer against heterologous challenge by other means e.g. 'infection and treatment' but perhaps not immunisation with attenuated stock (Jongejan *et al., 1*993b). Better prospects now appear to be with killed vaccine, but development of a broadly protective subunit vaccine may be possible.

It was interesting that virulence alone was not the only factor related to immunogenicity otherwise the Suswa isolate would have ranked top and perhaps the Marigat isolate would not have protected against the Baragoi isolate. These factors: density and others need to be investigated. The Kathiani 972 isolate also protected against the Baragoi isolate completely in the one way trial. Both these isolates might be suitable vaccine candidate and more manageable as they were both less virulent than the Baragoi isolate. However, more data is required on these cross immunity combinations.

The question arises as to when and how susceptible ruminants (sheep or cattle), should be immunised. It is the older and mature ruminants that have not had previous exposure (usually of exotic origin), that have been found to be most susceptible to clinical disease. These and young animals that are likely to become exposed as they grow older, are the ones that most need to be protected. Neonatal ruminants have been shown to have a brief period of innate resistance which is not related to the immune status of the dam (Neitz and Alexander, 1941; Uilenberg, 1971), although it can be influenced by the dams immunity (Deem *et al.,* 1996b). It has been shown that by the time ruminants are born they already possess a functional (able to respond to antigens), immune system though much weaker than mature animals (Tizard, 1987). This is because the neonate has not been primed and any infection has to be processed through the primary response (rather than anamestic) (Keith and McGuire, 1989; Tizard, 1987). The point of immunisation is to maintain or establish immunity in the herd so that disease incidence is avoided or kept on a low profile. Although Du Plessis, (1970, 1993) & Byrom *et al.,* (1993), did not observe neutralising effect on elementary bodies by antibodies to add to protective immunity, Byrom *et al.,* (1993),

observed that recovered serum prevented entry of *Cowdria* agent into endothelial cells *in vitro*. This may mean that antibodies to *Cowdria* might protect the neonatal endothelium while they facilitate uptake by phagocytes and other effector cells thus allowing the neonate to mount an immune response. It is possible that the inability to respond strongly constitutes the strength of the innate resistance in protecting against the effects of over production of IFN-γ and TNF which are known to be harmful when over produced (Dellanoy *et al., 1*993). This has created the theory that Cowdriosis is an immune disease because *Cowdria* elementary bodies can be detected for many days (Neitz *et al., 1*986c), before the clinical signs precipitate, and clinical signs usually appear at around the time the immune response is taking effect. Thus, Neitz and Alexander, (1941, 1945), successfully immunised young calves against heartwater while shielding them against clinical disease with innate resistance. It follows that preference should be made to immunise younger cattle (one to 2 months of age), but this matter requires further investigation as much variability about the upper age limit has been found by various workers including breed and exposure (Uilenberg, 1983). Innate resistance in young sheep and goats has also been found profitable for assisting in heartwater immunisation, but is variable to a lesser degree. This would reduce the risk and losses due to disease following immunisation and also in drug use. The demonstration that at least some of the calves in an endemic area become infected by their dams via the vertical route (Deem *et al., 1*996a), (although this appears not to have occurred in calves of immunised dams, (Camus 1987), vertical infection indicates the advantage there is in early immunisation. This can then be boosted through natural infection.

Until an improved and acceptable vaccine becomes available, ruminants will have to be immunised by the infection and treatment method as originally designed by Neitz and Alexander, (1941,1945) (or culture EBs). The guidelines for production and standardisation of vaccine have been laid down (Bezuidenhout, 1981; Oberem and Bezuidenhout, 1987b). A local isolate is to be preferred if live vaccine is to be used on account of the huge differences in immunogenicity and virulence that have been encountered by many workers with isolates of different geographical origin.

5.5 Conclusion

The different isolates induced qualitatively (and quantitatively), different immunologically responses that led to different levels of protection against each other. Because of this there is potential danger of heartwater infection to naive as well as recovered animals moving into and between endemic parts of the country. On the basis of this study it seems unlikely that mild strains will make effective vaccines. This study supports the contention that virulence is an important side effect of the protective response in line with the report of Du Plessis, (1982). The findings that loss of virulence during attenuation (Jongejan, 1991) may reduce the cross protection induced by the attenuated Senegal strain is apparent in the data of (Jongejan *et al.*, 1993b). From this study the most suitable characteristics for a potential vaccine were found with Baragoi (though virulent), and the Marigat and Kathiani 972 isolates but not the Suswa isolate are recommended for further work to develop a vaccine in Kenya.

Table 5.1: Plan of stabilates and sheep used for a two way cross-immunity trial.

Experiment	Group	Immunising stock	No. sheep receiving 1° inoculation	No. sheep receiving 2° inoculation	Heterologous challenge stock	No. sheep I ..... C^2	
1	1	Bamba St. 348	20	20 + $2c^1$	Kathiani 972	5	2
					Asembo	5	2
					Baragoi	5	2
					Suswa		
	2	Asembo St. 352	20	20 + $2c^1$	Bamba	5	2
					Kathiani 972	5	"
					Baragoi	5	"
					Suswa	5	"
2	3	Baragoi St. 349	20	20 + $2c^1$	Bamba	5	2
					Asembo	5	2
					Kathiani 972	5	2
					Suswa	5	2
	4	Suswa St. 341	20	20 + $2c^1$	Bamba	5	"
					Asembo	5	"
					Baragoi	5	2
					Kathiani 972	5	"

KEY

Groups - 1 & 2 = sheep in the first experiment, 3 & 4 sneep in the second experiment

I - immunised animals inoculated with 1 ml neat stabilate and treated with oxytetracycline (TLA) at 20 mg/kg body weight.

1° - treatment was given for this infection; 2° - treatment not given for this infection

C^1 -. naive sheep given 2 ml diluted stabilate (1/3 ml neat equivalent) and terminated *in extremis*

C^2 - naive sheep given 1 ml neat stabilate and terminated *in extremis* two animals were used for each challenge stabilate

" - where groups 1&2 and 3&4 share the same controls

Table 5.2a: The outcome of a one way challenge of recovered sheep with a heterologous stock

Column	2	3	4	5	6	7	8	9	10	11	12	13
Sheep #	**1° infection**	**stabilate**	**Treated**	**Challenge infection**	**stabilate**	**Incubation**	**Max. temp**	**Duration of fever**	**Day to death**	**Day to recovery**	**RI 2°**	**RI 1°**
35	Kath. 32	330	n	Suswa	327	9	41.4	2	10	-	51.4	11.0
63	Asembo	352	y	Suswa	342	12	41.5	4	15	-	50.7	(12.3)
20	Asembo	352	y	Baragoi	346	13	41.8	4	16	-	50.2	(21.8)
177	Asembo	352	n	Kiswani	331	13	41.8	4	16	-	50.1	14.5
58	Kath. 32	330	n	Kath. 972	327	11	41.7	6	16	-	49.5	7.5
62	Asembo	352	n	Isiolo	325	13	41.7	6	-	19	13.9	11.3
34	Bamba	304	n	Kath. 972	327	13	41.8	7	-	20	13.2	13.7
55	Bamba	348	n	Gal 233	305	14	41.2	8	-	22	12.9	12.9
33	Kiswani	TC EBs	y	Asembo	352	13	42.0	6	-	19	12.7	(7.8)
57	Bamba	348	n	Isiolo	325	12	41.0	7	-	19	8.2	6.7
61	Gal 233	305	y	Kath. 972	327	21	40.8	2	-	25	3.2	(8.5)
83	Bamba	348	n	Marigat	345	18	40	3	-	22	2.3	19.7
48	Marigat	311	n	Baragoi	346	11	41	1	-	12	0.8	12.3
38	Isiolo	325	y	Gal 233	305	NR	-	-	-	-	0.0	(8.8)
54	Suswa	341	y	Kiswani	331	NR	-	-	-	-	0.0	(5.0)
59	Baragoi	346	y	Suswa	342	NR	-	-	-	-	0.0	(8.5)
46	Kath. 972	327	y	Baragoi	346	NR	-	-	-	-	0.0	(11.6)
47	Marigat	345	n	Bamba	348	NR	-	-	-	-	0.0	14.7
183	Bamba	348	n	Kath. 32	333	NR	-	-	-	-	0.0	12.1
187	Bamba	348	n	Kath. 32	333	NR	-	-	-	-	0.0	16.9

KEY: n = no; y = yes; NR = no reaction; - = not applicable. Column 1, 2, 4, & 5 - from Table 4.2a & b; Column 12 - RI of sheep during challenge infection; Column 13 - RI of sheep during primary infection (treated).

Table 5.2b: The outcome of a one way challenge of recovered sheep with a heterologous stock: comparison of reaction indices

Column 2		3	4	5	6	7	8	9
Sheep #	1° infection	Treated	2° infection	RI 2°	RI m 1°	RI m 2°	dif.	pf
177	Asembo	n	Kiswani	50.1	45.0	46.9	-3.2	-7.0
20	Asembo	y	Baragoi	50.2	45.0	50.1	-0.1	-0.2
35	Kath. 32	n	Suswa	51.4	9.3	51.8	0.4	0.7
58	Kath. 32	n	Kath. 972	49.5	9.3	49.6	0.1	0.1
63	Asembo	y	Suswa	50.7	45.0	51.8	1.1	2.1
62	Asembo	n	Isiolo	13.9	45.0	47.8	33.9	71.0
33	Kiswani	y	Asembo	12.7	46.9	45.0	32.3	71.9
34	Bamba	n	Kath. 972	13.2	13.1	49.6	36.4	73.4
55	Bamba	n	Gal 233	12.9	13.1	48.8	35.9	73.6
57	Bamba	n	Isiolo	8.2	13.1	47.8	39.6	82.9
61	Gal 233	y	Kath. 972	3.2	46.2	49.6	46.4	93.5
83	Bamba	n	Marigat	2.3	13.1	47.7	45.4	95.2
48	Marigat	n	Baragoi	0.8	47.7	50.1	49.3	98.5
183	Bamba	n	Kath. 32	0	13.1	9.3	9.3	100
187	Bamba	n	Kath. 32	0	13.1	9.3	9.3	100
47	Marigat	n	Bamba	0	47.7	13.1	13.1	100
54	Suswa	y	Kiswani	0	51.8	46.9	46.9	100
38	Isiolo	y	Gal 233	0	47.8	48.4	48.4	100
46	Kath. 972	y	Baragoi	0	49.6	50.1	50.1	100
59	Baragoi	y	Suswa	0	51.1	51.8	51.8	100

KEY

Column 1&2 - Table 4.2a & b

Column 5 - RI 2° = RI of sheep during challenge infection

Column 6 - RI m 1° = median RI for isolate as determined in Chapter 4

Column 7 - RI m 2° = median RI for isolate in challenge infection

Column 8 - dif.= difference between the median RI for challenge isolate (column 7) and the RI of reaction to challenge infection (column 5)

Column 9 - pf = protection factor (section 5.2.6.2)

Table 5.3: The mean reaction index (RI), mean reduction in RI and the protection factor of groups of sheep, immunised and challenged with different isolates compared with control sheep.

	Immuniser									
	mRI		mRI		mRI		mRI		mRI	
Challenger	Asembo	p f (%)	Bamba	p f (%)	Baragoi	p f (%)	Suswa	p f (%)	controls	p f (%)
Asembo	2.2*	25.8(92.2)	11.7	15.9(57.6)	0.5	27.2(98.3)	12.3	15.3(55.3)	27.6	0(0.0)
Bamba	2.4	10.5(81.4)	0.8	12.1(93.6)	0.0	13.0(100)	0.3	12.6(97.5)	13.0	0(0.0)
Baragoi	45.5**	4.8(9.5)	46.4	3.8(7.6)	1.7	48.6(96.7)	9.5	40.8(81.1)	50.2	0(0.0)
Suswa	43.8	6.8(1.4)	41.5	9.1(18.0)	0.1	50.5(99.7)	0.3	50.3(99.3)	50.6	0(0.0)
Kath. 972	48.9	0.0(0.0)	42.2	6.7(14.1)	0.3	48.6(99.6)	2.1	46.8(95.9)	48.9	0(0.0)

KEY

mRI - mean reaction index

pf(%) - protection factor (reduction in mean RI as a percent of RI of control sheep)

2.2* - mean RI for homologous boost data underlined (20 sheep per group)

heterologous groups (not underlined) five sheep per group except ** where one sheep died before challenge.

Table 5.4: Severity score of reaction in homologous booster infections for four isolates. Numbers indicate the number of animals with each score of reaction severity/ category

Isolate/ category	Boost 1--2--3--4	Mean severity score	Controls (2) 1--2--3--4	Mean severity score	X(%)
Asembo	9 6 5 0	1.3	0 0 0 2	10	8.70 (87.0)
Bamba	16 3 1 0	0.4	1 0 1 0	2	1.65 (82.5)
Baragoi	11 3 6 0	1.4	0 0 0 2	10	8.65 (86.5)
Suswa	11 6 3 0	0.9	0 0 0 2	10	9.10 (91.0)

KEY

Reaction	Category	Score applied to each animal
none	1 - no reaction (fever never reaching 40°C cut off)	0
mild	2 - mild fever temperature not exceeding 40.5°C	1
severe	3 - high fever (≥40.5°C) and/or clinical sign	4
very severe	4 - death with or without clinical signs	10

X - protection level (value by which the mean reaction has reduced compared with the control sheep and percent of control sheep)

Table 5.5: Summary of animal response: severity of reaction (number of sheep) in different reaction categories, score of group and mean of severity score per animal due to heterologous challenge with different isolates.

Challenge isolate	Immunising isolate: Asembo 1 2 3 4	Score (group mean)	Bamba 1 2 3 4	Score (group mean)	Baragoi 1 2 3 4	Score (group mean)	Suswa 1 2 3 4	Score (group mean)	Controls 1 2 3 4	Score (group mean)
Asembo	-	na	1 1 2 1	19(4.8)	4 1 0 0	1(0.2)	0 0 5 0	20(4)	0 0 1 3	34(8.5)
Bamba	2 2 1 0	6(1.2)	-	na	5 0 0 0	0(0)	4 1 0 0	1(0.2)	0 1 3 0	13(3.3)
Baragoi	0 0 0 4*	40(10)	0 0 0 5	50(10)	-	na	0 0 5 0	20(4)	0 0 0 4	40(10)
Suswa	0 0 1 4	44(8.8)	0 0 0 5	50(10)	5 0 0 0	0(0)	-	na	0 0 0 4	40(10)
Kath 972	0 0 0 5	50(10)	0 0 1 4	44(8.8)	4 1 0 0	1(0.2)	1 3 1 0	7(1.4)	0 0 0 4	40(10)

KEY

Reaction	Category	Score applied to each animal
none	1 - no reaction (fever never reaching 40°C cut off)	0
mild	2 - mild fever temperature not exceeding 40.5°C	1
severe	3 - high fever (≥40.5°C) and/or clinical sign	4
very severe	4 - death with/without clinical signs	10

Challenge groups - five sheep per group each except * where one sheep died before challenge.
Controls - four sheep per group

Table 5.6: The mean reduction in severity score of sheep immunised and challenged with different isolates

Challenging isolate	Immunising isolate				Controls*
	Asembo (%)	Bamba (%)	Baragoi (%)	Suswa (%)	
Asembo	**8.7 (87.0)**	3.75(44.1)	8.3(97.1)	4.5(52.9)	8.5
Bamba	2.1(63.1)*	**1.65 (82.5)**	3.25(100)	2.25(69.2)	3.3
Baragoi	0(0.0)	0(0.0)	**8.65 (86.5)**	6(60.0)	10
Suswa	1.2(12.0)	0(0.0)	10(100)	**9.10 (91.0)**	10
Kath 972	0(0.0)	1.2(12.0)	9.8(98.0)	8.6(86.0)	10
Total(mean)*	75.1(18.8)	56.2(14.0)	395.1(98.8)	268.2(67.0)	-

KEY
mean protection of heterologous challenge
2.1(63.1)* - reduction in reaction severity score of sheep immunised with Asembo and challenged with Bamba = 2.1 (a 63.1% reduction in severity)
Controls* - severity score of control infections
Total(mean)* - against heterologous challenge
Homologous data - from Table 5.4 shown in bold Heterologous data - derived from Table 5.5

Table 5.7: Summary of serological response of immunised sheep: number of sheep with different titre patterns

	Immunisation and boost				Heterologous challenge			
Isolate	End of boost total +ve	Con. inc. ↗↗	Inc. plat. ↗→	Inc. dec. ↗↘	Total	Increase ↗	Decrease ↘	Plateaux →
Asembo*	20/20	5	8	5	6	3	0	3
Bamba	18/20	8	5	5	5	4	0	1
Baragoi	20/20	19	1	0	20	2	13	5
Suswa**	19/20	14	3	3	19	8	10	1

KEY
- con inc - continuous increase in titre from preinfection to post infection, and from post infection to boost infection
- inc plat - titre increase from preinfection to post infection but did not increase from post infection to boost infection
- inc dec - titre increase from preinfection to post infection then fell from post infection to boost infection

Note
- Increase or decrease considered only if titre was different by 5 units (from Appendices 5.1 & 5.2)
- some titres increased from preinfection to post infection below cut off level, then increase from post infection to boost infection reached the 70% cut off level (Baragoi (1), Suswa (3), and Bamba (1) sheep. Two other Bamba infected animals did not register any change in titre while one of the Suswa infected sheep (# 104) sera titre rose but did not reach cut off at any time.
- Asembo* - all sera present after infection, but one animal died and another was not tested at the end of boost in this group therefore apparent total 18!
- Suswa** - one sample not tested in heterologous sera

Table 5.8a: The median and range of incubation period for sheep immunised and challenged with different isolates

Isolate	Control	Immuniser			
Challenger	Naive	Asembo	Bamba	Baragoi	Suswa
Asembo	10(7-16)	na	13(11-13)	13(-)	13(12-14)
Bamba	16.5(14-19)	22(15-23)	na	none	16(-)
Baragoi	9(7-11)	10(9-11)	10(9-10)	na	14(12-15)
Suswa	8(7-10)	9(6-9) R	8(0-10) R	22(-)	na
Kat. 972	10(9-10)	10(10-12)	11(10-13)	21(-)	14.5(14-20)

Table 5.8b: The median and range of fever duration in sheep immunised and challenged with different isolates

Isolate	Control	Immuniser			
Challenger	Naive	Asembo	Bamba	Baragoi	Suswa
Asembo	7(3-15)	na	3(3-4)	2(-)	7(5-10)
Bamba	7(3-9)	3(2-5)	na	(-)	1(-)
Baragoi	4.5(3-6)	4(3-5) R	4(3-6)	na	6(5-7)
Suswa	3(1-6)	2(1-8) R	3(0-6) R	1(-)	na
Kat. 972	4(4-5)	4(3-5) R	3(3-6) R	1(-)	2(1-6)

Table 5.8c: The median and range of days to death in sheep immunised and challenged with different isolates

Isolate	Control	Immuniser			
Challenger	Naive	Asembo	Bamba	Baragoi	Suswa
Asembo	15(14-22)	na	14(-)	(-)	(-)
Bamba	21*	(-)	na	(-)	(-)
Baragoi	13(12-14)	13.5(11-14)R	13(12-15)	na	(-)
Suswa	10(10-12)	9(9-10) R	11(9-15) R	(-)	na
Kat. 972	13(13-14)	14(12-16) R	14(13-14)	(-)	(-)

KEY (for Tables 5.8a, b & c)
naive - obtained from Table 4.2a R - note shift
* - only one death occurred with this stabilate

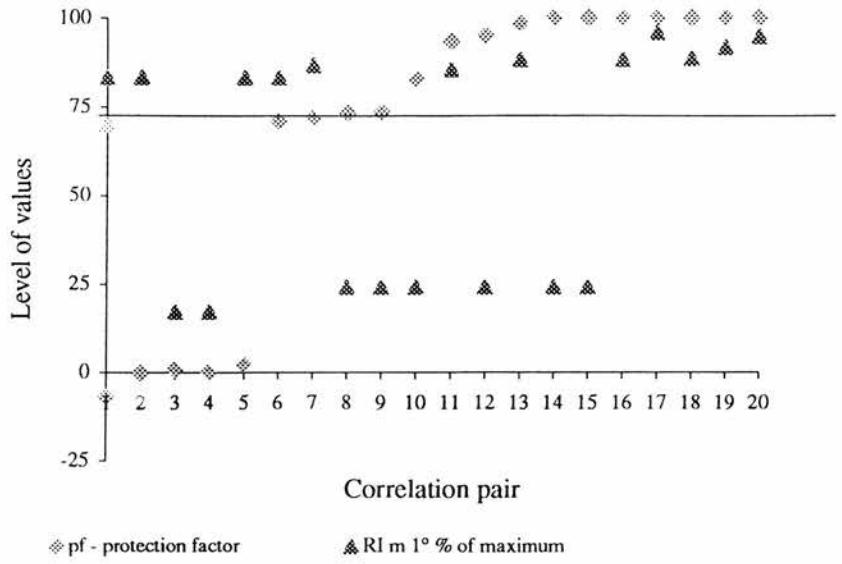
Table 5.9: Summary of significance level between inhibition level of sera collected at the end of the different phases of the cross immunity experiment.

Sera	P value				
compared by T test	**Asembo**	**Bamba**	**Baragoi**	**Suswa**	**Interpretation**
pre infection/all*	0.001	0.001	0.001	0.001	significant rise in antibody
post infection/post boost	ns	ns	0.001	0.05	significant rise in some
Post infection/post heterologous	0.001	0.05	ns	ns	significant rise in some
post boost- post heterologous	0.01	ns	0.01	ns	significant rise and fall

KEY

*comparison of preinfection serum with (all) the other 3 post sera (all highly sinificant)

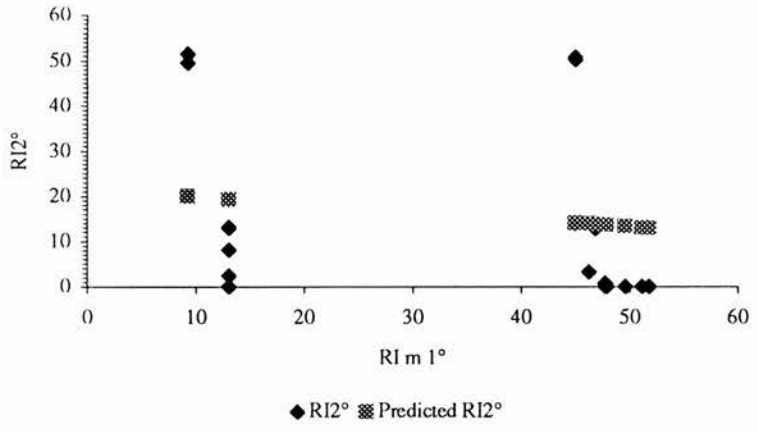
- pre infection serum collected prior to infection
- post infection serum collected day 28 post infection except Bamba due to longer incubation period
- post boost serum collected day 28 post homologous boost inoculation except Baragoi and Suswa collected 21 days post boost
- post heterologous serum collected 28 days post heterologous inoculation



KEY

———— 70% 'cut off': sheep with pf below this did not survive virulent challenge

Figure 5.1a Correlation of protection factor (pf) with the reaction index of immunising isolate expressed as a percentage of maximum RI (correlation coefficient = 0.133) (derived from Table 5.2b).



RI m 1° Line Fit Plot

Figure 5.1b Regression of reaction index of challenge isolate (RI2°) on the reaction index of the immunising isolate (RI m 1°) (derived from table 5.2b).

CHAPTER SIX

INFECTIVITY OF KENYAN *COWDRIA* ISOLATES TO BALB/C MICE

6.1 Introduction

Discovery of the first mouse pathogenic *Cowdria,* the Kumm strain (Du Plessis and Kumm, 1971), revolutionised the methods of study of *Cowdria.* It led to the attainment of a laboratory model to study many aspects of cowdriosis relatively cheaply. This included screening drugs (McHardy and MacKenzie, 1987), pathology (Prozesky, 1987b), pathogenesis, development and immunology (Byrom *et al. 1*993). Whereas the study of pathogenicity for mice became the first pointer that *Cowdria* stocks can have significant antigenic differences between them, earlier workers considered such stocks as atypical *Cowdria* (Ramisse and Uilenberg, 1971; Uilenberg, 1977, 1981; Van Winklehoff and Uilenberg, 1981; Uilenberg *et al., 1*983). As more murinotropic *Cowdria* were discovered it became evident that in nature, stocks probably embrace a broad spectrum of murinotropism (Du Plessis *et al., 1*989; Du Plessis *et al.,* (1992b). A review which placed known isolates of *Cowdria* into different categories of murinotropism has been compiled (MacKenzie and McHardy 1987). At one end of the spectrum are highly mouse pathogenic stocks such as Kumm (Du Plessis and Kumm, 1971); Kwanyanga (MacKenzie and Van Rooyen, 1981); Nonile (MacKenzie and McHardy, 1987), the Welgevonden (Du Plessis, 1985b), a Nigerian isolate (Okoh *et al.,* 1986), and Crystal Springs (Byrom *et al., 1*993). There are isolates which infect mice but are non pathogenic to mice such as Ball 3 (Haig, 1952); Senegal (Wassink *et al., 1*990), and Comoro (Du Plessis *et al., 1*989). It was appreciated only in the 1980s that the Ball 3 first transported in mice (Haig, 1952), was maintained by a subclinical infection in the mice. The majority of *Cowdria* isolates encountered by scientists do not cause clinically observable effects in the mice.These have been grouped in the 'non-pathogenic or non infectious' isolates (MacKenzie and McHardy, 1987). The ability of some stocks to cause disease in suceptible ruminant after many passages in mice, led to their being classified as non pathogenic, by MacKenzie and McHardy, (1987), while others were unrecoverable after even one passage. Du Plessis *et al.,* (1989) showed that the infectious, non pathogenic Ball 3 and Comoro stocks, elicited an antibody response in carrier mice, which they defined as a fourth category of *Cowdria.* Among the inapparent isolates, MacKenzie and McHardy, (1987), passage through the mice could be demonstrated a serological

response (Du Plessis *et al., 1*989) while the remaining isolates were undetectable even by antibody response. MacKenzie and McHardy, (1987) found the infectivity and pathogenicity depends on the mouse strain to a degree. The route of inoculation was important, all isolates were only infective by the intravenous route except the Kumm stock which was also infective by the intraperitoneal route (MacKenzie and McHardy, 1987).

The ability of mice to develop specific immunity to various murinotropic strains has been demonstrated by homologous challenge (Du Plessis, 1982; MacKenzie and Van Rooyen, 1981), as well as heterologous challenge (Stewart, 1987b, 1989). As in ruminants, homologous challenge did not cause death in any mice. However, some isolates did not always protect against other isolates. Death was the outcome in all, some or none of the challenged mice depending on the isolate used to infect first and which was used to challenge (Stewart, 1989). The Kwanyanga isolate protected mice against Welgevonden, whereas in the reverse situation, Welgevonden failed to protect more mice against Kwanyanga challenge. Kwanyanga and Welgevonden protected against Nonile challenge, and Kumm infected mice were susceptible to all other isolates (Stewart, 1989) .

Immunity in mice was demonstrated to be cell mediated (Du Plessis, 1982; Stewart, 1987b, 1989). Transferring immune spleen cells pre-incubated with the organism for 20 minutes, to susceptible mice protected the recipient mice. But a similar experiment with cells from non immune mice resulted in infection and death. Antibody did not seem to play a protective role (Du Plessis, 1982). Stewart (1987b), suggested that lymphokines might have a role in cell mediated immunity in the mouse by activating macrophages to destroy the agent.

With the exception of the Kumm stock which forms large colonies in mouse peritoneal macrophages, it is difficult to demonstrate *Cowdria* infection in mice. Infected mouse peritoneal macrophages facilitated the development of the immunofluorescent test for cowdriosis (Du Plessis, 1981b). Colonies of the Kwanyanga stock in mice have been seen in histological sections by Wassink *et al.*, (1990).

In this study, *Cowdria* isolates were tested for their infectivity for mice. An isolate was selected to immunise Balb/C mice which induced an antibody response, and the mice were then challenged with a mouse pathogenic stock. The outcome was compared with cross immunity results in sheep using the same pair of isolates.

The objective of this study was to determine the infectivity of *Cowdria ruminantium* isolates made from different geographic locations in Kenya, and to compare the protection afforded by a mouse infective but non-lethal *Cowdria* against a mouse pathogenic isolate.

6.2 Materials and methods

6.2.1 *COWDRIA*

Eleven Kenyan isolates whose infection in sheep have been described in Chapter 4, were tested for mouse infectivity. Fresh blood collected from a reacting sheep into EDTA anticoagulant or a cryopreserved blood stabilate (Section 3.2.6) were used.

6.2.2 MICE

These were eight week old, female Balb/C mice as described in Section 2.3.

6.2.3 SHEEP

Sheep used in these studies were 5 to 12 month old, heartwater naive, *Cowdria* seronegative to the cELISA test (Section 2.5.1)

6.2.4 *MODUS OPERANDI*

The mice were inoculated intravenously with 0.2 ml of blood into a tail vein. A minimum of five mice were injected i/v per group except for the Suswa isolate in which four mice were injected.

They were monitored daily by clinical inspection until day 28 post inoculation (pi) then euthanased. Impression smears of lung, liver, spleen, kidney and crush smears of the cerebral cortex were prepared from mice found dead or which were euthansed in extremis. They were stained with Giemsa and examined for the presence of *Cowdria* colonies in the endothelial cells of blood vessel and macrophages in the mouse organs.

6.2.5 INFECTIVITY TEST FOR *COWDRIA* IN MOUSE ORGANS

Mice that survived greater than 28 days and which did not develop clinical reactions were sacrificed by dislocating the cervical vertebrae and blood drawn from the heart into a heparinsed syringe using a G26 hypodermic needle. Spleens were dissected out aseptically into heparinised (50 IU per millilitre), Glasgow's Minimum Essential Medium (GMEM medium) (GIBCO BRL), or PBS. The spleens were transferred to a tissue homogeniser (Bellco Biological glassware), after extra fat was dissected off and crushed gently but firmly in 3 ml GMEM culture medium or PBS. Cells were harvested by pouring off the supernatant into a universal tube. The inoculum mouse organ homogenate (MOH), was composed by mixing the blood collected from the mice and the supernatant (Jongejan *et al., 1*990). The mixture (4 to 5 ml), was kept cool on crushed ice and the entire pool inoculated i/v into one sheep. The sheep was monitored clinically and by recording of daily rectal temperature until day 28 post inoculation. Brain crush smears of sheep that died were examined for *Cowdria* bodies by the method of Purchase, (1945).

6.2.6 ONE WAY CROSS IMMUNITY TRIAL IN MICE

Twelve Balb/C mice were inoculated intravenously with 0.2 ml Bamba blood stabilate using a gauge 25 to G27 hypodermic needle and a, 1 millilitre syringe. On day 28 the immunised mice and, 15 unimmunised (control), mice were challenged using 0.2 ml of the Asembo stock inoculated intravenously. Mice that had not been successfully infected, were challenged 5 days later (= 34 pi). Mice were monitored daily by clinical inspection and behaviour noted until day 28 post inoculation (pi). Mice that became sick were euthanased and post mortem record made of lesions that were present.

6.2.7 SEROLOGY

Serum was collected from mice as detailed in Section 2.6. This was tested by the indirect Enzyme Linked Immunosorbent Assay (iELISA), Section 2.6.2. Sheep infected with MOH were checked for seroconversion by testing serum collected on day 0 and day 28 post infection using the iELISA described in 2.6.1.

6.3 Results

6.3.1 MURINOTROPISM OF KENYAN ISOLATES

Two Kenyan isolates were mouse pathogenic, the Asembo and the Baragoi isolates, which each caused 66% mortality in inoculated mice (Table 6.1). Seven isolates (Bamba, Suswa, Kathiani 32, Isiolo, Kiswani, Galana 229, Galana 233), were mouse infective only, of which one (Kiswani) could be passaged in MOH (Jongejan *et al., 1990*) to sheep. Two isolates (Kathiani 972 and Marigat) were non infective.

6.3.2 MANIFESTATION OF *COWDRIA* INFECTION IN MICE

Clinical signs were only seen in the mice infected with two isolates (Asembo and Baragoi) that caused mortality. Four out of six mice inoculated i/v with Asembo blood died, and deaths occurred on days 12 and 13. However, clinical signs of anorexia, piloerection, huddling and hyperventilation were seen only in one mouse; the other mice were found dead in the morning. Two mice inoculated with less than 0.2 ml blood also died (Table 6.2), one on day, 14 and the other on day, 15.

Four out of six mice infected with 0.2ml Baragoi blood died, and deaths occurred on day, 14 and 15. Only two of them showed clinical signs similar to the mice in the Asembo group. One mouse inoculated with less than 0.2 ml, died on day seven from this isolate.

The mice infected with all the other 9 isolates did not show any clinical signs or deaths that could be associated with *Cowdria* infection. However, one mouse infected with Kathiani 972 with no heartwater signs at post mortem (haemorrhagic enteritis) died on day 18.

6.3.3 POST MORTEM FINDINGS

The main lesions found in affected mice (Tables 6.3a & b), were hydrothorax, enlarged liver and pneumonitis. Hydrothorax fluid in most mice was between 0.1 and 1.2 ml, and had a similar appearance to that found in sheep with heartwater. Mild pneumonitis and pale enlarged liver were regularly found but were not striking. The gall bladder was usually full and prominent (in some mice abdominal organs were bile stained yellow), and in some mice, mild congestion /erythema of the intestines was accompanied by a tarry orange content. Frank haemorrhage was not seen. The spleen

showed at best only a mild gross pathology and kidneys were sometimes quite pale.

One mouse infected with the Isiolo isolate, had a strong mucosal haemorrhage in the caecum: it was euthanised on day 29 pi, a day after being bled for serum when it was found dull and anorexic. Another mouse infected with the Kathiani 972 was sacrificed on day, 18 when it developed paralysis of the hind quarters. It was found to be suffering from a haemorrhagic enteritis of the small intestines (Table, 6.1). Both deaths, were considered to be due to causes other than heartwater possibly coccidiosis.

6.3.4 MICROSCOPIC EXAMINATION FOR INFECTION IN MICE

Impression smears made from the internal organs of mice found dead or euthanased in extremis were prepared as described in Section 6.2.4 and examined for *Cowdria* under the microscope (Leitz Leibovitz, Germany). Cells from affected mice appeared to be active in general and degeneration from pathological or post mortem changes seemed to set in quickly. There was prevalent vacuolation in cells from the liver and kidney while rickettsia were generally absent or could not be conclusively identified. No definitive rickettsial organisms were seen in any of the impression smears and presence of *Cowdria* organisms in brain endothelial or macrophage cells could not be confirmed. This examination was discontinued when it became clear that *Cowdria* could not be identified satisfactorily in such preparations early in the study.

6.3.5 SEROLOGICAL EVALUATION OF INFECTION

Sera collected prior to infection and 28 days post infection were submitted to the iELISA (Section 2.6.2) to assess the antibody response. Figure 6.1a. is a distribution frequency for uninfected mice from which the 95% cut off level (OD reading of 0.143), was used to determine a positive serological response in the mice (Appendix 6.1). A high proportion of the mice inoculated with the apathogenic isolates indicated an antibody rise post infection to a level that was well above the cut off except for two stocks (Marigat and Kathiani 972 stocks). The rates of seroconversion fell into three groups, the high proportion group included: Galana 233 (5 out of 5); Isiolo (6 out of 6); Bamba (5 out of 6) and Kathiani 32 (4 out of 5) An intermediate group included Suswa (2 out of 4); Galana 229 (2 out of 6) and Kiswani (2 out of 6). The

third group comprised the none or low proportion of seroconverters which included Kathiani 972 (0 out of 4) and Marigat (0 out of 5). There was sero conversion in the survivors of the pathogenic Asembo (2 out of 2) and Baragoi (1 out of 2). Additionally seroconverters occurred in mice which received less than 0.2ml inoculum intravenously (table 6.2).

The OD values for seropositive mice ranged between 1 and 5 times the cut off value (cut off = 0.143). Most positive OD values were low positive (OD values less than 0.2, close to the cut off). Medium positives (OD between 0.2 and 0.4), were observed in Galana 233, Asembo and Baragoi infected mice. High positives (OD between 0.5 to 0.8), were observed in Galana 229 (which exceeded the positive control serum OD of 0.75), and Isiolo infected mice.

6.3.6 RESPONSE OF SHEEP INOCULATED WITH MOUSE ORGAN HOMOGENATES (MOH)

There was no clinical signs and no seroconversion in the sheep inoculated with mouse organ homogenates except for one isolate. Only the sheep inoculated with organs from mice infected with Kiswani isolate contracted typical heartwater and died on day, 15. It was confirmed through the brain crush smears which had colonies in endothelial cells of brain capillaries.

6.4 Cross immunity trial

Mice that were immunised with the Bamba stabilate (12 mice) did not show any clinical signs just as mice tested earlier with Bamba infected blood. This stabilate was used concurrently in the cross immunity trial in sheep and they became infected. Evidence for infection were obtained by performing serology.

On heterologous challenge, clinical signs occurred in some, and deaths occurred in all the mice. Immunised as well as non immunised control mice showed similar clinical signs and all died. The immunised group tended to live marginally longer (median 13; range 11 to 14 days post challenge), overlapping with the control (non-immune) mice (median 11; range 11 to 13 days post challenge) (Table 6.3b).

6.4.1 PATHOLOGY IN FATAL CASES

They all exhibited post mortem lesions attributable to heartwater. Pathology in the immunised mice appeared to be slightly reduced. There was less hydrothorax in the immunised mice than the non-immunised mice (median value 0.3 ml versus 0.5 ml respectively). The heart lesions in the immune mice were more pronounced than in the non-immune mice, whereas the spleen tended to be enlarged in the non-immune mice. Fewer mice had kidney lesions in the immune mice than in the non-immune mice (Table 6.3b).

6.4.2 SEROLOGY

The only way to confirm that the mice had become infected with the Bamba isolate was through the serological response. Most mice immunised with the Bamba isolate did not seroconvert to the cut off level (OD = 0.143). However, 5 out of 12 'immunised' mice had between > x1 to > x3 increase in OD value of their preinfection levels. Only in two out of 12 mice did antibody exceeded the cut off (interpreted as low positive) (Table 6.4). Therefore two mice seroconverted according to the cut off. The serum of one mouse was not available and the results of one mouse was out of ranges at preinfection and therefore both results were excluded. However, there exists a chance that these two mice could have been stimulated making a possible total of seven 'stimulated' mice. There was no post challenge serum to compare as there was no survivors.

6.5 Discussion

6.5.1 MURINOTROPISM OF KENYAN ISOLATES

In this study *Cowdria* was inoculated intravenously (and subcutaneously by default), which categorised Kenyan isolates into two groups at the level of infectivity: the pathogenic and the non pathogenic. The two used routes of infection (i/p and i/v), in the mouse affects the infectivity of *Cowdria* stocks differently (Mackenzie and McHardy, 1984; Du Plessis, 1982), and this has been used to discriminate them, but the intravenous route is the most successful in most host species for most stocks of *Cowdria*. The Kumm stock infects mice equally well by the intravenous as well as the intraperitoneal route (Du Plessis and Kumm, 1971).

Use of the mouse model was the first to indicate that there might be significant antigenic variation between different *Cowdria* isolates and has unravelled the fact that strains of *C. ruminantium* vary considerably with respect to their infectivity (Mackenzie and Van Rooyen, 1981; McHardy and MacKenzie, 1987). The implication is that different isolates probably have a different host range each. For example, the Kumm stock is pathogenic for mice and sheep while being almost non-pathogenic for cattle (Du Plessis, 1982). In this study one isolate, the Asembo, was found to be similar to the Kumm; highly pathogenic for mice and moderately pathogenic for sheep but is not pathogenic for cattle (Rumberia, R. pers. coms). The other mouse pathogenic isolate, the Baragoi, was found to be highly pathogenic to all three species just as is the Welgevonden from South Africa (Du Plessis, 1985b).

Typical clinical signs as recorded by Prozesky and Du Plessis (1987) and typical post mortem lesions as described by Du Plessis (1975), Prozesky and Du Plessis (1984), Prozesky (1987b) and Prozesky and Du Plessis (1985) were observed. Microscopic examination was not successful as found by MacKenzie and Van Rooyen (1981) and Wassink *et al.* (1990) .

6.5.2 SEROLOGICAL EVALUATION OF INFECTION AND IMMUNITY

Antibody response has been assessed and found to be present even in the absence of clinical signs (Du Plessis *et al., 1*989). According to Tizard (1987), a conversion from seronegative to seropositive or a fourfold rise in the titre signifies a serological response. After Serology, the Kenyan isolates were grouped into three categories of mouse infectivity. The Asembo and Baragoi which caused deaths in the mice and stimulated substantial antibody response in some of the survivors, can be considered to be the first category. Seven isolates, the Bamba, Kathiani32, Suswa, Isiolo, Kiswani, Galana 229 and the Galana 233 invoked humoral responses in a good proportion of the mice or relatively strong responses in a few mice without causing a clinical manifestations - they fall into the second category. Two isolates, the Marigat and Kathiani 972 stimulated a low or no humoral response in the majority of the mice and were thought to be refractile - forming the third category.

After immunisation with the Bamba stabilate, it appeared that the mice had a lower

stimulation than when febrile blood was used (Appendix 6.1k). One reason for the difference in magnitude of the serological response could be that mildness of the Bamba isolate is due to its naturally low titres that develop in infected animals (suggested by neutrophil and endothelial culture findings). Therefore the stabilate started with a low titre which was reduced by storage: Du Plessis and Malan (1988), found that infective blood lost an average of 0.73 log of infectivity after storage in liquid nitrogen. Another reason is perhaps the fact that the original blood makes 90% of stabilate. Therefore, stabilate performed only marginally worse than the fresh blood which had resulted in low positives. Neither those that showed some serological response, nor the ones that did not show a serological response were protected against the Asembo isolate.

The Asembo isolate did not kill sheep immunised with the Bamba with the exception of one sheep that was found not to have seroconverted and was therefore not immune. As observed by Uilenberg (1983), pathogenesis may be due to a quite different mechanism in mice and ruminants, which was also implied by the difference in virulence of the two isolates in mice (and sheep) in this study.

6.5.3 RESPONSE OF SHEEP INOCULATED WITH MOUSE ORGAN HOMOGENATES (MOH)

The fourth category of Kenyan *Cowdria* was found among the seven isolates that stimulated only antibody response in Balb/C mice. The Kiswani was the only Kenyan isolate in this category because fatal cowdriosis was transmitted to a sheep inoculated with mouse organ homogenates, (Table 6.5). Haig (1955), maintained organisms in mice for 90 days without deleterious effects and was thus the first to infect mice with a non-pathogenic strain of *Cowdria*. Du Plessis (1982), was able to recover the organisms from the liver, lung and myocardium 365 days after infection, but only 30 days from peritoneal macrophages although these disappeared in immune mice after challenge (Du Plessis, 1982). The Kiswani would appear to fall in the same category as the Ball 3 (Haig, 1952); the Mara stocks (Haig, 1952), a Malagasy strain (Ramisse and Uilenberg, 1971), a Sudanese strain (Abdel-Rahim and Shommein, 1978), and the Senegal strain (Jongejan *et al.*, 1988), which are mild or inapparent strains in mice.

The Kiswani isolate had been maintained in the mice for more than 30 days while the other six isolates, were eliminated from the organs used to prepare the homogenates.

The fact that only one sheep inoculated with MOH became infected with *Cowdria* suggests that among the non pathogenic isolates, only the Kiswani remained viable and hence infectious to sheep. The other non virulent isolates were probably eliminated at various stages of infection, and the antibody response reflects the relative ability (extent of infection), of the isolates to infect Balb/C mice before they were controlled. Those in which antibody response was not detectable could be due to the fact that the inoculum was greatly diluted and the organisms eliminated early or before they started to multiply. The situation may be different with different strains of laboratory mice and also in the field (Stewart, 1989).

6.6 Conclusion

This work has demonstrated Kenyan stocks of *C. ruminantium* to differ markedly in their murinotropism and fall into four categories (Table 6.5). Albeit no wild rodent has been found naturally infected with *Cowdria,* Mason and Alexander (1940), as well as MacKenzie and McHardy (1987), were able to infect wild rats with *Cowdria* and therefore implicated wild rodents in the maintenance and transmission of heartwater in the field. Two mouse pathogenic and one non-pathogenic Kenyan *Cowdria* might possibly be involved in the infection of feral rodents at large through larval or nymphal tick transmission and thus contribute to the epidemiology. However the other isolates might have a totally different infectivity with other laboratory mice and wild rodents.

Table 6.1: The infectivity of Kenyan *Cowdria* isolates for Balb/C mice: intravenous infection with 0.2 ml fresh blood

Source sheep no.	Isolate	Number of mice infected	No. mice (days to dev. clinical signs)	Deaths in group (days to death)	iELISA response no. positive
52	Asembo	6	1(13)	4(12, 12, 12, 13)	2/2*
44	Baragoi	6	2(14)	4(14, 14, 14, 15)	1/2*
27	Isiolo	6	1(29)[1]	1(29)	6/6
51	Galana233	5	-	-	5/5
34	Bamba	6	-	-	5/6
35	Kathiani32	5	-	-	4/5
41	Suswa	4	-	-	2/4
33	Kiswani	6	-	-	2/6
30 & 31	Galana229	4+2	-	-	2/6
972	Kathiani 972	5	-	-[3]	0/4*
29	Marigat	5	-	-	0/5

KEY

( )[1] mouse dull, anorexic, cuddled, shivering (put down in extremis) a day after serum had been collected

* results only for surviving mice

iELISA. - 95% cut off level for a negative population of mice used (OD 0.143)

Table 6.2: The infectivity of Kenyan *Cowdria* isolates for Balb/C mice: infection with - < 0.2ml fresh sheep blood which may not have been intravenous

Source sheep no.	Isolate	Number of mice infected	No. mice (days to dev. clinical signs)	No. deaths in group (days to death)	iELISA response # positive
52	Asembo	6	1(14)	2(14, 15)	1/4*
44	Baragoi	4	-	1(7)	1/3*
35	Kathiani32	3	-	-	3/3
41	Suswa	19	-	-	15/19
51	Galana233	9	-	-	7/9
27	Isiolo	7	-	-	4/7
34	Bamba	6	-	-	1/5**
972	Kathiani972	7	-	-	1/7
33	Kiswani	2	-	-	0/2
29	Marigat	5	-	-	0/5
30 & 31	Galana229	3+8	-	1(10)[4]	0/10

KEY

* results for only surviving mice

1/5** one serum lost

( )[4] diarrhoea around the tail found clinical signs - generally anorexia, piloerection, breathing rapidly and curled up or hurdled together,

iELISA. - 95% cut off level for a negative population of mice used (OD 0.143)

Table 6.3a: Post mortem findings in mice infected with *Cowdria*: infectivity testing

Isolate	Mouse	Hydro thorax	Abdominal fluid	Lung lesion	Liver enlargement	Gall bladder	Spleen	Stained gut content	Stained omental fat
Baragoi	c5cm4	+	-	+	+	-	+	+	-
	c4cm3	+	+	-	+	+	+	-	-
	c5cm3	+	-	+	+	+	+	-	-
	c4cm6	+	-	-	+	+	+	-	-
	c5cm5	-	-	+	+	-	-	-	-
Asembo	c2dm1	+	-	+	+	+	-	-	-
	c1dm2	+	-	+	+	-	-	-	-
	c2dm4	+	-	+	+	-	-	-	+
	c2dm6	+	-	-	-	-	-	+	-
	c1dm4	+	-	-	+	-	-	-	-
Crystal Spring	c1em4	+	-	+	+	+	+	+	-
	c1em2	+	-	+	+	+	+	+	-
	c1em5	+	-	+	+	+	+	+	-
	c2em1	+	-	+	+	-	+	+	-
	c2em4	+	-	+	+	-	+	+	-

NB

Crystal Spring - mice infected with this stock (to produce immune serum) added for comparison

Table 6.3b:Post mortem lesions of mice infected with *Cowdria*: cross-immunity trial

Mouse	Day of death	Hydro thorax fluid (ml)	Lung lesion	Heart pallor	Spleen enlarge -ment	Kidney pallor	Liver enlarge-ment	Fullnes s of gall bladder	Stained git content
Bamba immunised									
c1fm4°	11	0.42	+	+	+	+	+++	++	-
c2fm1°	14	na	+	++	+/-	-	++	++	+
c2fm3°	14	na	+	+	+/-	-	++	++++	++
c2fm4°	13	0.5	+	++	+/-	-	+++	+++	+
c2fm5°	14	0.3	+	+	+/-	-	+	++	-
c3fm4°	12	0.9	+	++	+	+/-	+++	++++	+
c3fm5°	14	0.1	+/-	+	+/-	-	++	++	-
c3fm6°	13	1.2	+	++	+	-	+++	+++	+
Control mice									
c4fm1	13	0.4	+	+	+/-	-	+	++	-
c4fm3	12	0.5	+	++	+/-	+	+++	++++	+
c4fm5	13	0.9	+	+	+	+	+++	++++	+
c5fm2	11	0.5	+	+	++	+	+++	++	-
c5fm3	11	0.5	+	+	++	+	+++	++	-
c5fm4	11	0.3	+	+	+	+	+++	++	-
c5fm5	11	0.5	+	-	++	-	+++	++	-

KEY

c1fm4°- an immunised mouse; c4fm1- a non-immunised mouse

na -not available as mouse had been mauled by others; git - gastrointestinal tract

+,-,++,+++, - lesions present, absent, and relative degree

++++ - bile had stained abdominal organs due to leakage from gall bladder.

Lesions: lung - emphysema, oedema and hepatisation in portions

Table 6.4 - One way cross-immunity between Bamba and Asembo in Balb/C mice: mice infected with blood stabilate

Mouse immunised naive	Pre-Bamba immunisation	Post- Bamba immunisation	Sero conversion	Post-Asembo challenge	Protection
c8em1	0.965*	0.156	+	⇧	nil
c8em2	0.078	0.27	+	⇧	nil
c9em3	0.038	0.124	-	⇧	nil
c9em5	0.054	0.13	-	⇧	nil
c1fm4	0.087	nd	na	⇧	nil
c2fm1	0.15	0.128	-	⇧	nil
c2fm3	0.121	0.143	+	⇧	nil
c2fm4	0.113	0.082	-	⇧	nil
c2fm5	0.115	0.129	-	⇧	nil
c3fm4	0.09	0.117	-	⇧	nil
c3fm5	0.142	0.079	-	⇧	nil
c3fm6	0.093	0.098	-	⇧	nil
c3em2	0.064	nd	na	⇧	nil
c3em3	0.048	nd	na	⇧	nil
c3em4	0.046	nd	na	⇧	nil
c7em1	0.025	nd	na	⇧	nil
c7em2	0.051	nd	na	⇧	nil
c7em3	0.035	nd	na	⇧	nil
c7em4	0.031	nd	na	⇧	nil
c7em5	0.037	nd	na	⇧	nil
c4fm1	0.077	0.106	-	⇧	nil
c4fm3	0.087	0.089	-	⇧	nil
c4fm5	0.101	0.156	+	⇧	nil
c5fm2	0.143	0.093	-	⇧	nil
c5fm3	0.109	0.112	-	⇧	nil
c5fm4	0.08	0.074	-	⇧	nil
c5fm5	0.123	0.083	-	⇧	nil

KEY

⇧ - had died nd - not done na = not applicable

iELISA OD values for mice* - extreme outlier, ignored

negative pool OD = 0.037 positive control = 0.75 cut off value = 0.143

Table 6.5 The classification of Kenyan isolates in terms of infection to mice

Mouse pathogenic	Infectious non-pathogenic carrier state	Infectious non pathogenic antibody response	Non infectious
Asembo	Kiswani	Galana 229	Kathiani 972
Baragoi		Galana 233	Marigat
		Kathiani 32	
		Isiolo	
		Suswa	
		Bamba	

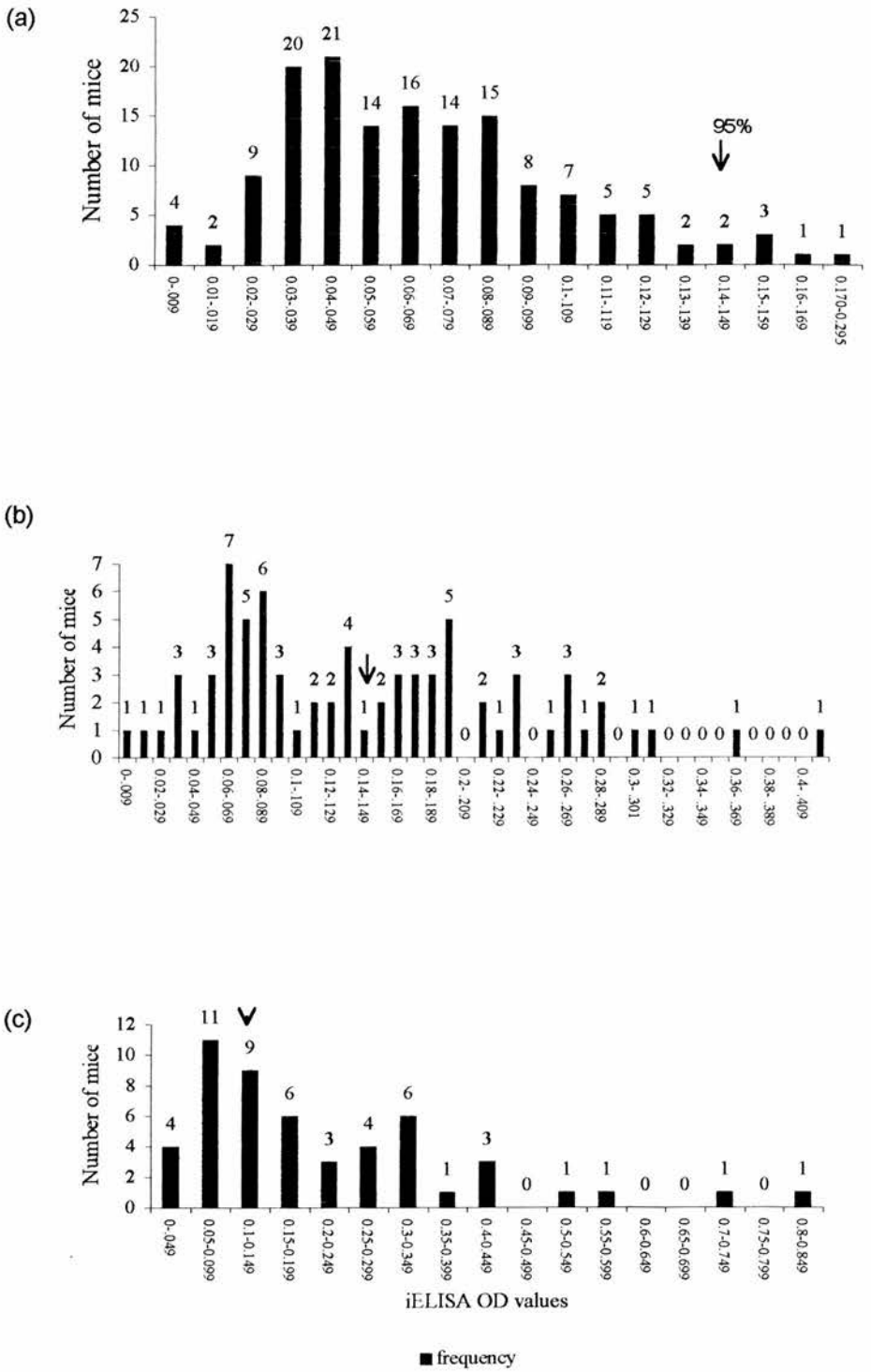


Figure 6.1 Frequency distribution iELISA OD values for a negative mouse population, mice infected with < 0.2ml (34 out of 76) and with 0.2ml Cowdria infected blood (29 out of 51 mice)

KEY → position of positive cut off value (OD = 0.143)

CHAPTER SEVEN

ATTEMPT TO DETERMINE SUSCEPTIBILITY OF TWO SPECIES OF KENYAN *AMBLYOMMA* TICK VECTORS TO INFECTION WITH TWO *COWDRIA* ISOLATES.

7.1 Introduction

Amblyomma ticks belong to the only genus of tick vectors known to transmit heartwater naturally (Bezuidenhout, 1987). There are ten proven African vectors of this species; *Amblyomma variegatum, A. hebraeum, A. lepidum, A. astrion, A. gemma A. pomposum, A. tholloni, A. sparsum, A. cohaerens* and *A. marmorium,* and three American species *A. maculatum, A. cajannense* and *A. dissimile,* which are experimental vectors, (Uilenberg and Camus, 1993). Adults of *Amblyomma* ticks have preference for certain hosts, and because of this, not all *Amblyomma* ticks are equally important in field transmission to domestic ruminants, (Petney *et al.*, 1987; Petney, and Horak, 1988). Whereas different species have been reported to be predominant in different zones, (Uilenberg, 1983; Norval *et al.*, 1992), two species *A. variegatum* and *A. hebraeum* predominate overall. *A. variegatum* is the most abundant vector of heartwater in Eastern and Central Africa (Walker and Olwage, 1987) whereas *A. hebraeum* takes this role in South and Southern Africa. Apart from West Africa (Gueye *et al.,* 1993), there is evidence that *A. hebraeum* may be a more effective transmitter of heartwater because more clinical cases have been observed where *A. hebraeum* is the main vector, than in areas where *A. variegatum* is the main vector, (Norval *et al.*, 1992; Asselbergs *et al.*, 1993). The level of seropositivity is also higher where *A. hebraeum* predominates over *A. variegatum,* (Asselbergs *et al.*, 1993; De Vries *et al.*, 1993). *A. hebraeum* was equally susceptible to *Cowdria* isolates obtained from Southern Africa as well as from other parts of Africa, whereas *A. variegatum* ticks were not equally susceptible (Mahan *et al.*, 1995; Peter *et al., 1*995). This difference in susceptibility of *A. variegatum* ticks to infection with different isolates, may provide a reason for the reputed reduced seriousness of heartwater experienced in areas where *A. variegatum* is the predominant vector, in comparison to areas where *A. hebraeum* is the predominant vector, for example the reduced impact of heartwater in the Caribbean compared to Southern African regions. It is likely that other heartwater vectors may also be found to have a difference in susceptibility to different strains of *Cowdria.* This would affect heartwater epidemiology in each area.

In Kenya, *A. variegatum* is found in many districts and has an overlapping distribution

with *Rhipicephalus appendiculatus*, the vector of East Coast fever, while *A. gemma* is found in drier areas but overlaps with *A. variegatum* in certain environments, (Walker, 1974). Activities directed at the control of ECF are likely to affect other ticks and the disease that they cause such as *Amblyomma* and associated diseases. The aim of this work was to investigate the susceptibility of the two main Kenyan vectors, *A. variegatum* and *A. gemma* to *Cowdria* which had been isolated from the two species.

The objective of this study was to determine the infection rate in *A. variegatum* and *A. gemma* ticks when co-fed on sheep infected with a heartwater blood stabilate obtained from either *A. variegatum* or *A. gemma* ticks.

7.2 Materials and methods

7.2.1 EXPERIMENTAL DESIGN

Sheep were infected with the Bamba and Asembo isolates, *Amblyomma variegatum* and *A. gemma* ticks applied on two occasions during the febrile response in separate bags. The ticks were examined for infection by PCR. Before use, conditions were first optimised and evaluated using control *Cowdria* DNA and with extracts from blood and ticks (experiments 1 to 11).

7.2.2 *COWDRIA* STOCKS

Two *Cowdria* isolates: the Asembo and Bamba isolates obtained from *A. variegatum* and *A. gemma* ticks at the shores of the lake Victoria and at the Kenya coast respectively were used. The reduced virulence of these isolates was considered useful to the placement and feeding of ticks on reacting animals.

7.2.3 SHEEP

Six susceptible sheep, as described in Section 2.2 were used. Three sheep were inoculated with the Asembo and three sheep inoculated with Bamba blood stabilates. A vial of each stabilate was thawed rapidly in a 37°C water bath and 1 ml inoculated intravenously into the jugular vein of three sheep for each stock. The sheep were monitored clinically and post mortem was performed as described in Section 2.4.

They were not treated during high fever so as to enable as many ticks as possible to feed without interference with transmission mechanisms. Clotted blood for serum was collected on days 0 and 28 from the sheep.

All three sheep inoculated with Asembo stabilate developed signs of infection and all reacted with fever at the expected time. Two out of three sheep inoculated with one millilitre of the Bamba blood stabilate contracted heartwater but the febrile reactions in these animals were later than expected while no temperature reaction occurred in the third sheep. There was seroconversion in the cELISA test to the Welgevonden soluble antigen in all survivor sheep except the Bamba inoculated sheep that did not become febrile (no. 102). Two sheep died following Asembo infection and had pathology consistent with that of cowdriosis, and had colonies in the endothelial cells in the crush smears of the brain cortex according to the method of Purchase, (1945).

7.2.4 TICKS

Clean nymphs of *A. variegatum* and *A. gemma* were used to pick up the infection from the sheep by patch feeding.

TICK COLONIES

The two species, *A. variegatum* and *A. gemma.* were laboratory maintained. *A. variegatum* was established at the National Veterinary Research Centre (NVRC) Muguga in 1994. Ticks in this colony were collected from Narok district as engorged females and the larvae obtained, maintained through the stages on rabbits. A group of 100 nymphs each was fed on two susceptible sheep to test for transmission. Other ticks from the same batch were used for infectivity studies since no heartwater was transmitted by either of the groups.

A. gemma ticks were obtained from two sources: from an NVRC colony that had been established in the late seventies or early eighties from the field and maintained on rabbits only, and the ILRI clean colony which was established from ticks collected at Kibogo Field site for the Kenya Veterinary Laboratories (Embakasi) in the 1980s. The ILRI ticks were maintained alternately on rabbits and steers by feeding the adult ticks on cattle and the intermediate stages (larvae and nymphae) on rabbits.

TICK INFECTION

A summary of sheep used , when ticks were applied and when they dropped off is shown in Table 7.1. A patch was shaved on the back of the sheep large enough to put two (sheep 91 &102) or four (sheep 176, 177, 183 & 187) body patches. The sites were then washed with soap and water to remove grease and allowed to dry. Cotton bags were glued to the patches using Evostick™ and allowed to set and fumes to evaporate. Five hundred ticks were applied in separate bags at 2 days interval starting six days before fever was expected to begin. Sheep # 91 and 102 had ticks added to the same patch but in all the other sheep, the second batch of ticks was placed in a separate patch. Tubes containing the nymphs were opened and placed in the patch and secured with tape. The applications were timed so that nymphs were predicted to engorge at the start of fever (on day six for Asembo and day 8 for Bamba infections). The tubes were removed after the ticks had crawled out and attached to the sheep back. Engorged ticks were collected into separate tubes for each patch daily until all the ticks had engorged and dropped. They were incubated at 28°C and 85% relative humidity until they moulted into adults and hardened, (Bailey, 1960). They were then transferred to an 18°C incubator until needed for dissection.

7.2.5 ASSESSMENT OF TICK INFECTION RATES

For each day of the pyretic phase, ten ticks of each species, (5m + 5F), which had dropped off an animal were dissected. Ten ticks fed on uninfected sheep were also dissected to act as negative controls.

7.2.6 TICK DISSECTION

Ticks were handled asseptically. A tick was placed on a new sterile petri dish kept bottom up and held down with one finger. The tick legs and posterior end were sliced off with a sterile scalpel and internal organs extruded by firmly stroking down the tick dorsum using the scalpel blade held at an angle. The organs were picked up with the tip of a sterile disposable hypodermic needle and placed into a sterile Eppendorf tube and transferred onto crushed ice. A new pair of gloves was used whenever contamination of gloves was suspected. The samples were frozen at -70 C until

testing.

7.2.7 NUCLEIC ACID EXTRACTION.

Dissected guts were freeze thawed twice (frozen in a -70 C freezer, thawed in a 37 C water bath) and digested in 200µl proteinase K (PK) buffer (see Appendix 2.2) containing 2.5µg /ml proteinase-K, incubated at 37 C overnight then at 56 C for a further 1 hr.

Extraction of DNA from digested material was carried out by the Phenol chloroform extraction method, (Sambrook *et al.*, 1989). Viz.:

After spinning at maximum speed (12,000- 14,000 rpm) on a microcentrifuge (Eppendorf centrifuge 5414), the top aqueous layer was removed, transferred to a new tube and an equal volume of reagent added each of three times: after the first spin, Tris buffer (pH 8) equilibrated phenol was added. Phenol:chloroform isoamyl alcohol (1:1) was added the second time, and finally chloroform:isoamyl alcohol (24:1) was added the third time. Each time the tube was inverted gently but thoroughly to mix the extract and reagent before spinning. The extracted nucleic acids were topped up with TE buffer to a final volume of 260 µl (approx.) and stored at -20 C until ready for PCR amplification and blotting.

DETERMINATION OF TICK DNA CONCENTRATION

DNA concentration was estimated by obtaining the OD reading at a wavelength of 260λ on a spectrometer (Unicam 8625 UV/VIS spectrometer) and the reading converted using the factors:-

a 1 OD = 50µg/ml (Sambrook *et al.*, 1989)

b dilution factor of stock sample made

Therefore [DNA] µg /ml = OD reading x a x b

7.2.8 PCR TO DETECT *COWDRIA RUMINANTIUM* IN *AMBLYOMMA* TICKS

7.2.8.1 PRIMERS CHOICE, SEQUENCE AND PRODUCT LENGTH

Two sets of primers were used to amplify *Cowdria*: the first were based on the pCS20

DNA probe, the AB128/AB129 primers, (Waghela *et al.*, 1991; Mahan *et al.*, 1992), and primers based on the 16S rRNA gene sequence, HE and EC primers, (Section 2.8.1). The primer sequences and theoretical specificity have been given in Table 7.2. The 16S rDNA primers comprised of the following pairs of primers, HE1(cr)/HE3(l), HE1(cr)/HE3(s), HE1(cow)/HE3(l), HE1(cow)/HE3(s), which all amplify a DNA fragment of 388 bp and HE2/HE3(s) which amplifies a 352 bp product because HE2 is located at the 3′ end of HE1(cr). Universal primers, EC11/EC12 amplified a 767 bp product. A set of 16S rDNA primers, Avar-F1/Avar-R2, that could amplify tick DNA was designed to work under similar conditions as the AB primers. The setting up of the PCR reaction has been described in section 2.8.3 and format of the constituent reagents shown in Appendix 2.2. The detection of PCR products is described in section 2.8.4, and the precautions taken in Section 2.8.5.

CYCLE CONDITIONS

The amplification conditions for all the primer combinations used in this study are given in Table 7.3. Pre-amplification protocols were part of the thermocycling programme unless the master mix needed to be added to a denatured sample.

Experiment 1 To establish the sensitivity of AB128/129 primer for control *C. ruminantium* (Welgevonden) DNA..

Aim

To determine if AB128/129 primers amplify a product of 279 bp in control *Cowdria* DNA.

Method

Cowdria ruminantium (Welgevonden) DNA prepared from tissue culture EBs was used as *Cowdria* control DNA. A series of ten-fold dilutions was prepared from a stock containing 10 ng/µl to give the standard dilutions to test in these experiments: Five microlitre of each of the dilutions; 1 ng/µl, 0.1 ng/µl, 10 pg/µl, 1 pg/µl, a 100 fg/µl, 10 fg/µl, 1 fg/µl was tested as per protocol using 5µM primer AB128/AB129. Samples were denatured by heating at 96 C on the amplifying block for 10 minutes, cooling on ice and pulsing down before master mix was added and amplification carried out to a hot start of 90°C using programme 'A' on Table 7.3.

Experiment 2 To optimise PCR sensitivity of AB128/AB129 primers to amplify control *Cowdria* DNA dilutions in three concentrations of magnesium chloride and in presence of tick extract

Aim

To determine if chelating agent used to preserve tick samples affects the ability of the AB128/AB129 primers to amplify (*C. ruminantium* Welgevonden) *Cowdria* because tick extracts were preserved in TE buffer which contains EDTA, a chelating agent for $MgCl_2$. Taq buffer provides 10 x 3 mM $MgCl_2$ a concentration which can be changed by adding an exogenous solution of $MgCl_2$. This test was to investigate if the EDTA in the TE buffer might affect the efficiency of the AB primers to amplify the samples.

Method

The concentration of $MgCl_2$ was adjusted to a final concentration of 3 mg/µl, (the protocol standard), 4 mg/µl and 5 mg/µl in three master mixes. The three mixes contained extracted control tick pool DNA. While the control PCR contained 3 mg/µl $MgCl_2$ and no tick extracts, (as in experiment 1). Five microlitres of the standard *C. ruminantium* (Welgevonden) DNA dilutions were thermocycled in the presence of 5µl of undiluted tick extract in the four master mixes; pre-amplification protocol no 2) and programme 'A' Table 7.3.

(Outcome Figures 7.1 and 7.2)

Experiment 3 To show the ability of AB128/AB129 primers to amplify control *Cowdria* DNA dilutions in the presence of Avar-F1/Avar-R2 primers and Avar-F1/Avar-R2 primer to amplify tick pool DNA in the presence and absence of AB128/129 (*Cowdria*) primer.

Aim

To determine the ability of the two sets of primers to amplify their specific product in the presence of the other set of primers under the same conditions.

Method

Standard dilutions of cultured *C. ruminantium* (Welgevonden) control DNA and ten-fold dilutions of *A. variegatum* tick pool DNA were tested in the presence of the two

sets of primers that amplify each of them (5μM AB128/129 primer, and 2μM Avar-F1/Avar-R2 primers). 3μM magnesium chloride was used. (Pre-amplification protocol no 2) and programme 'A' (Table 7.3)).

(Outcome Figures 7.3).

Experiment 4 To determine the ability of Avar-F1/Avar-R2 primers to amplify tick pool DNA and AB128/129 primers to amplify Asembo and Bamba Qiagen extracted *Cowdria* DNA.

Aim

To confirm the detection limit of Avar-F1/Avar-R2 primers to detect tick DNA and determine whether AB128/129 primers detect Asembo or Bamba DNA under the same conditions.

Method

Ten-fold dilutions of *A. variegatum* tick pool DNA were tested in duplicate sets using 2μM Avar-F1/Avar-R2 primers. Asembo and Bamba extracted by Qiagen column were tested using 5μM AB128/129 primers, and PCR performed by pre-amplification protocol no. 2 and programme 'A,' (Table 7.3).

(Outcome, Figures 7.4)

Experiment 5 To test the amplification of DNA from tick extracts of *C. ruminantium* (Bamba) isolate by AB128/AB129 primers in 4μM Magnesium Chloride.

Aim

To determine the proportion of infected ticks by PCR following tick feeding on a Bamba infected sheep, infected with *Cowdria.*

Method

All selected tick extracts from ticks fed on the Bamba infected sheep were tested using 5μM AB128/129 primers. $MgCl_2$ was used at 4μM concentration. Control DNA (Welgevonden EBs) 1 ng/μl, was used as a positive control, with sterile MilliQ water as the reagents control. Samples were denatured before addition of the master mix (Pre- amplification protocol no 2), and amplified using programme 'A' (Table

7.3)

(Outcome, Figures 7.5)

Experiment 6 To determine the sensitivity of AB128/AB129 primers for different *C. ruminantium* isolates.

Aim

To determine the ability of the AB128/AB129 primers to amplify 279 bp product in different Kenyan isolates. This was undertaken because of failure of earlier attempts to amplify Asembo isolate.

Method

Qiagen extracts of Asembo, Bamba, Kathiani 972, and Baragoi isolates from blood stabilate, Senegal and Isiolo tissue culture, and two tick extracts that had produced a product before with the AB128/AB129 primers were tested. Concentration of $MgCl_2$ was at 4μM. Control DNA (Welgevonden) 1 ng/μl, was used as a positive control with sterile MilliQ water as the reagents control. Samples were introduced into the master mix under oil, denatured by one cycle, (pre-amplification protocol no. 3) and thermocycled on programme 'A' (Table 7.3)

(outcome, Figures 7.6)

Experiment 7 To determine the sensitivity of HE1(cr)/HE3(s), and HE2/HE3(s) primers for amplification of Asembo infection in tick extracts and Qiagen extracted Asembo and Bamba DNA.

Aim

To compare the ability of specific HE1(cr)/HE3(s) primers and less specific HE2/HE3(s) primers to produce a product in replicate samples of Asembo tick extracts and Qiagen extracts of Asembo and Bamba DNA, to determine suitability for use of these primer set for both Asembo and Bamba infection.

Method

Test tick extracts from Asembo infected sheep, Qiagen extracts of Asembo and Bamba DNA from blood stabilate, and two tick extracts that had produced a product

with the AB128/AB129 primers were tested. Concentration of $MgCl_2$ was at 4μM. Positive control DNA, Welgevonden EBs (1 ng/μl), and sterile MilliQ water were included as the reagents control. Samples were introduced into the master mix under oil, denatured by pre-amplification protocol no. 3) and amplified using programme 'B'. (Table 7.3).

(Outcome, Figures 7.7)

Experiment 8 To test the amplification of DNA from different *C. ruminantium* isolates by HE1(cow)/HE3(l) primers and Asembo DNA by HE2/HE3(s) at the standard annealing temperature.

Aim

To determine if HE1(cow)/HE3(l) primers (which are specific for *Cowdria*) amplify Asembo and Bamba isolates (and other Kenyan isolates) under conditions established previously and to determine if Asembo DNA is amplified under the same conditions by HE2/HE3(s) primers, which have broad *Ehrlichia* specificity.

Method

Qiagen extracts of different isolates from blood stabilate, and two tick extracts that had produced a product with the AB128/AB129 primers were tested. $MgCl_2$ was used at 4μM concentration. Control DNA (Welgevonden) 1 ng/μl, was used as a positive control, with sterile MilliQ water as the reagents control. Samples were introduced into the master mix under oil, denatured by one cycle of pre-amplification protocol (no. 2) then run on programme B (Table 7.3).

(Outcome, Figures 7.8)

Experiment 9 Amplification of DNA from different *C. ruminantium* isolates by HE1(cow)/HE3(s), HE2/HE3(s) and HE1(cow)/HE3(l) primers at a reduced annealing temperature.

Aim

To determine if there is improvement in the ability of PCR using the specific HE1(cow)/HE3(s), and HE1(cow)/HE3(l) primers and Ehrlichial primers HE2/HE3(s) to amplify different Kenyan *Cowdria* isolates when annealing

temperature is reduced.

Method

Qiagen extracts of different isolates from blood stabilate, and two tick extracts that had produced a product with the AB128/AB129 primers were tested. $MgCl_2$ was used at 4µM concentration. Control DNA (Welgevonden EBs) 1 ng/µl, was used as a positive control, and sterile MilliQ water as the reagents control. Samples were introduced into the master mix under oil, denatured by one cycle, protocol no. 3) and amplified using programme 'C' (Table 7.3).

(outcome, Figures 7.9)

Experiment 10 To test the amplification of DNA from different *C. ruminantium* isolates by HE1(cow)/HE3(s), primers in a nested PCR with EC11/EC12 primers.

Aim

To determine if nested PCR provides an improvement in the sensitivity of the HE1(cow)/HE3(s), primers for different *Cowdria* isolates by first amplifying *Cowdria* DNA using non-specific EC11/EC12 primers.

Method

Qiagen extracts of different isolates from blood stabilate, and two tick extracts that had produced a product with the AB128/AB129 primers were tested. $MgCl_2$ was used at 4µM concentration. Control DNA (Welgevonden EBs) 1 ng/µl, was used as a positive control and sterile MilliQ water used as the reagents control. Reagents were prepared at half volume for the EC primers. PCR was performed on programme 'E' (Table 7.3). Two microlitres of each reaction product were transferred to a corresponding tube of PCR reagents containing HE1(cow)/HE3(s) primers and thermocycled on programme 'G', (Table 7.3)

(outcome, Figures 7.10)

Experiment 11 To determine the ability of nested PCR to produce a product when EC11/EC12 primers are used in a 37 cycle initial PCR, followed by PCR with HE1(cow)/HE3(s) primers.

Aim

To attempt to overcome the difficulty in amplifying Asembo DNA by increasing the number of cycles in the first phase of the nested PCR using EC primers (37 cycles, Anderson *et al.*, 1992), under reduced annealing temperature for these primers.

Method

Qiagen extracts of different isolates from blood stabilate, and two tick extracts that had produced a product with the AB128/AB129 primers were tested. Concentration of $MgCl_2$ was at 4μM. Control DNA (Welgevonden) 1 ng/μl, was used as a positive control with sterile MilliQ water as the reagents control. Reagents were prepared at half volume with the EC primers. After completion of thermocycling with EC11/EC12 primers (Programme 'F'), 2μl of product from each reaction were transferred to a corresponding tube of PCR reagents prepared to amplify with the HE1(cow)/HE3(s) primers. Samples were introduced into the master mix under oil, and amplified on the thermocycler, pre-amplification protocol no. 3), followed by programme 'H,' (Table 7.3).

(Outcome, Figures 7.11)

Experiment 12 To determine the infection rate in Asembo tick extracts by nested PCR by using EC11/EC12 primers in the first reaction and HE1(cow)/HE3(s), primers in the second reaction.

Aim

To determine the number of tick extracts infected with *Cowdria* obtained from Asembo infected sheep after amplifying the complete PCR (37 cycles) with EC primers

Method

All tick extracts from ticks fed of Asembo infected sheep were tested. Concentration of $MgCl_2$ was at 4μM. Control DNA (Welgevonden) 1 ng/μl, was used as a positive control with sterile MilliQ water as the reagents control. Reagents were prepared at half volume with the EC primers (to avoid reagent wastage and cost). After completion of thermocycling with EC primers (Programme 'F'), 2μl of product from

each reaction were transferred to a corresponding tube of PCR reagents prepared to amplify with the HE primers. Samples were introduced into the master mix under oil, and amplified on the temperature cycler programmed to denature by one cycle (Pre-amplification protocol no. 3), then thermocycled on programme 'H' (Table 7.3).

(Outcome in Figures 7.12)

7.3 Results

7.3.1 DETERMINATION OF THE SENSITIVITY OF DIFFERENT PRIMERS FOR AMPLIFICATION OF ASEMBO AND BAMBA DNA IN TICKS

7.3.1.1 THE SENSITIVITY OF AB128/129 PRIMERS FOR *COWDRIA*

AB128/129 primers amplified a 279 bp product in control DNA, (*C. ruminantium* Welgevonden EBs) up to 1 pg/µl (the 5th dilution) (Figure 7.2) in the presence and absence of constant tick pool DNA in the standard (3µM) concentration of $MgCl_2$. The sensitivity of AB primers was still 1 pg/µl in the presence of tick pool and 5µM MgCl2. The detection limit of AB128/129 primers improved 100 fold to 10 fg/µl in the presence of tick extract and 4µM MgCl2 concentration, (Figures 7.1 & 7.2). All subsequent PCR were carried out with this concentration of $MgCl_2$.

AB128/AB129 primers amplified a 279 bp product in control (*C. ruminantium* Welgevonden) dilutions in the standard (3µM) concentration of $MgCl_2$ in the presence of Avar-F1/Avar-R2 up to 100 fg/µl (5th dilution) (Figure 7.3). This meant that there was not significant PCR inhibition by the tick extract and also that the Avar-F1/Avar-R2 primers were compatible with AB128/AB129.

The AB128/AB129 primers yielded a 279 bp product in Qiagen extracted Bamba DNA from blood stabilate but not Qiagen extracted Asembo DNA, (Figure 7.4). It was decided to use the AB128/AB129 primers to determine the infection rate in the Bamba infected ticks.

7.3.1.2 THE SENSITIVITY OF AVAR-F1/AVAR-R2 TO DETECT TICK DNA

The *Amblyomma* primers, Avar-F1/Avar-R2, amplified a 240 bp product in tick DNA pool in the presence and absence of AB128/AB129 primers indicating that they were

compatible with the AB primers, (Figures 7.3 & 7.4) They amplified tick DNA in one extra dilution in the presence of AB primers. The reason for this improved detection limit was not determined but could be the physical presence of other large molecules creating spatial stability for the tick primers to amplify the tick DNA.

7.3.1.3 DETECTION OF BAMBA *COWDRIA* ISOLATE DNA IN TICKS

Following the detection of Qiagen extracted Bamba DNA from blood at 4μ molar $MgCl_2$, (Figure 7.2), AB128/AB129 primers were used to test all the Bamba ticks under the most sensitive conditions for these primers. Bamba tick extracts (72) were tested in two PCR experiments using the AB128/AB129 primers, (and the 4 μmolar $MgCl_2$ concentration). A 279 bp product of *Cowdria* DNA was detected in two ticks. Tick sample no. 284, (Figure 7.5), and number 340 (not shown) were amplified to produce a faint product. These products were not detected when diluted further (Figure 7.6) using the AB128/AB129 primers. Use of the AB128/AB129 primers did not result in detection of *Cowdria* DNA in any of 64 Asembo infected ticks (not shown). DNA for the Asembo strain was amplified to produce a faint 279 bp product from a Qiagen extract from blood stabilate, (Figure 7.6). The two AB128/AB129 primers positive ticks also gave a weak 352 bp product in PCR with HE2/HE3(s) primers, (Figure 7.9), confirming infection with an *Ehrlichia* or *Cowdria*.

7.3.2.1 THE SENSITIVITY OF HE PRIMERS TO DETECT *COWDRIA*

Attention was turned to the 16S rDNA primers to find primers that would amplify Asembo DNA and test the Asembo infected ticks for infection rate. Use of HE2/HE3(s) in PCR amplified a 352 bp product in the Asembo DNA under identical (standard) annealing temperature, (60°C). The HE1(cr)/HE3(l) primers in PCR amplified a 388 bp product in the control Welgevonden DNA, (Figure 7.7).

The specificity of the HE primers was improved by substituting HE1(cow) in the place of HE1(cr). Use of HE1(cow)/HE3(l) primers in PCR amplified a 388 bp product in control DNA but not in any of the Kenyan isolates, whereas the PCR using the HE2/HE3(s) primers produced a 352 bp product in Asembo Qiagen extract in the same PCR. This indicated that use of the HE1(cow)/HE3(l) primers were not

sensitive enough to Asembo or other Kenyan isolates at this annealing temperature, (Figure 7.8). Sensitivity of PCR using three sets of HE primers HE1(cow)/HE3(l), HE2/HE3(s) and HE1(cow)/HE3(s) was greatly increased when the annealing temperature was reduced from 60°C to 52°C, (Figure, 7.9). However the specific primers did not detect the two AB128/129 primer positive ticks which were detected faintly by the HE2/HE3(s) primers in the same PCR.

7.3.2.2 EC11/EC12 PRIMERS AND NESTED PCR

EXTRACTS FROM ASEMBO STABILATE (Qiagen extracted)

Qiagen extracted Asembo DNA, together with Welgevonden control, Senegal, and the two AB128/129 primer positive ticks were amplified in a nested PCR for 10 cycles using the EC11/EC12 primers, then amplified with the HE1(cow)/HE3(s) specific primers. The 767 bp product was not visible after the first stage in any of the samples, but a strong 388 bp product was produced from aliquots of those reactions in the second reaction, (Figure 7.10) in Asembo, Senegal and control (Welgevonden) DNA. The 'Bamba' infected ticks which had been positive with AB128/129 primers remained negative in both steps. Reducing the annealing temperature for the EC11/EC12 primers, and amplifying the complete PCR cycles yielded a faint to moderate 767 bp product in the Asembo, Kathiani 972, Isiolo and Baragoi DNA and a strong 388 bp products from their aliquots, but Control (Welgevonden) and Senegal DNA also had 388 bp products, (Figure 7.11) although their 767 bp first stage product was not visible. Therefore the nested PCR was considered to be sensitive enough to test the Asembo infected tick extracts.

7.3.2.3 DETECTION OF ASEMBO *COWDRIA* DNA IN TICKS

Asembo tick extracts were, tested using nested PCR (Figure 7.11). However, none of the 130 Asembo tick extracts produced any visible product. The control Welgevonden EBs and Qiagen extracted Asembo DNA, both resulted in 767 bp, and 388 bp products, (Figure 7.12a & b).

7.4 TICK INFECTION EXPERIMENT

7.4.1 TICK APPLICATION TO INFECTED ANIMALS

The five hundred nymphal ticks applied on a particular day/patch engorged and dropped off over a period of 3 to 7 days (and all the 1,000 ticks from a sheep dropped off in 5-11 days). Drop off began five days post application for the *A. gemma* ticks and 6 days post application for *A. variegatum* ticks. The tick drops started gradually, came to a peak then tapered off, but the harvest of ticks that dropped off each day was low compared to the number of nymphs applied (and expected) due to a factor that could not be determined. *A. variegatum* as a species appeared to be more affected in this regard than *Amblyomma gemma* and on one sheep, only one *A. variegatum* tick out of five hundred ticks applied in one patch and two in the other patch were obtained compared to 46 and 498 *A. gemma* ticks respectively. This precluded analysis of ticks from this sheep (no. 187) but enough ticks were obtained from five of the six sheep; three Asembo infected sheep (91, 176 and 177) and two Bamba infected sheep (nos. 102 & 183). The ticks from sheep 187 and 102 both inoculated with the Bamba isolate were excluded from the study because as explained above, there was insufficient ticks to compare for sheep 187 and sheep 102 failed to become infected. However, ticks from one sheep one infected with Asembo (no. 177) and another infected with Bamba (no. 183) were studied by selecting ten ticks of each species of ticks from each day from parallel patches, for a comparison of infection rates. Control ticks were obtained from clean ticks from the laboratory colony fed on two heartwater naive and seronegative sheep (nos. 8 and 104).

7.4.2 DNA EXTRACTS

Five hundred and ninety nine ticks from seven sheep (nos. 91, 102, 176, 177, 183, 8, & 104) were dissected and nucleic acid extracted from them. Extracts from an equal number of ticks were selected from the drops of the two sheep by matching for the number of the two tick species and equal sex ratio for ticks that dropped on a particular day, (Table 7.1). Where there was less than the ten ticks of one species available, the number assessed was limited to the lesser number in the drop pair. Two

hundred and eighteen *Amblyomma* ticks were thereby selected for their gut extracts to be amplified by PCR for *Cowdria*.

The individual DNA concentration of the 218 selected tick extracts, (Table 7.1) was determined by spectrometry (Unicam 8625 UV/VIS spectrometer) at wave length 260λ (Table 7.4) The median DNA concentration was 1395µg/ml (range 725-1680µg/ml) in the *A. variegatum* control ticks from sheep 8 & 104, it was 1197.5µg/ml (range 840-1720µg/ml) in the *A. variegatum* ticks dropping from sheep no. 177, and 1252.5µg/ml (range 800-1720µg/ml) in *A. variegatum* ticks dropping from sheep no. 183. The DNA concentration was 1186.25µg/ml (range 445-2085µg/ml) in *A. gemma* ticks from sheep no. 177, and 1260µg/ml (range 1015-2030µg/ml) in *A. gemma* ticks from sheep no. 183. The majority of tick extracts had around 1200µg/ml. Six uninfected *A. variegatum* ticks with the highest DNA concentration were pooled to create a negative control. It contained a calculated concentration of 1482µg/ml tick DNA.

7.4.3 INFECTION RATE IN TICKS

Out of 72 ticks from Bamba infected sheep 2 were positive, one male *A. gemma* and one female *A. variegatum*. None of the 130 ticks from Asembo infected sheep were positive (Table 7.5).

7.5 Discussion

It was not possible to determine the more susceptible species of *Amblyomma* to infection with *Cowdria* between *A. variegatum* and *A. gemma*. This was because the infection rate of ticks in this study was very low and was not considered a good indicator of infection rate between the two species. The unexpected shift in temperature reaction in the Bamba sheep, plus the low tick harvests led to fewer overall number of ticks being infected. Two out of seventy two ticks which had fed on a sheep infected with the Bamba isolate were positive on PCR with primers AB128/AB129 and HE2/HE3(s). To determine if nested PCR could increase sensitivity and detect other infected ticks, a nested primer set (EC11/EC12,

(Anderson *et al.*, 1992) followed by internal set HE1(cow)/HE3(s)) were tested with *Cowdria* DNA, Kenyan isolates including the tick extracts which gave positive results with AB128/AB129. The nested PCR was found to increase the sensitivity of the HE primers for Asembo but not for Bamba. When the annealing temperature was reduced to 45°C and the number of cycles increased to 37 for the EC11/EC12 primers in the nested PCR strong 388 bp products were observed in Welgevonden EBs control, Asembo, Kathiani 972, Isiolo, and Baragoi Qiagen extracts of DNA from stabilate or tissue culture but not with 2 ticks putatively infected with Bamba. This would imply that the two positives with AB128/129 were false positive or the nested PCR was false negative. Watson (1993), found the potential of the PCR based on the 16S rRNA gene was limited by the apparent variation of *C. ruminantium* stocks at the HE1 variable region in the 16S rRNA gene, that is why HE1(cow) was designed: to cope with variation between Senegal sequence and Crystal Spring sequence. Considering the small amount of DNA that may have been present in the original sample, it is probable that the nested PCR was false negative due to inability to anneal considering base sequences differed from those of the primers. The positive ticks comprised one male *A. gemma* tick which dropped off two days before fever and the second, a female *A. variegatum* tick which dropped off two days after fever had started in sheep no. 183. as detected by two different primers AB128/AB129 (Figure 7.5), and HE2/HE3(s) (Figure 7.9). The fact that both species of ticks became infected means that they may both have a role in the infectious cycle of the Bamba isolate in the field. The Bamba isolate originated in *A. gemma* ticks but a few *A. variegatum* were observed in the area during these studies.

A nested PCR was developed that could detect Asembo strain DNA, Welgevonden and Asembo Qiagen extract from stabilate (positive controls) in the nested PCR but there were no *Cowdria* infected ticks among those fed on Asembo infected sheep as detected by the nested PCR using HE and EC primers. The infection rate of ticks in this experiment seem to confirm the findings of those workers who have encountered low infection rate in the field. For example, Du Plessis et al (1992b) found five percent infection rate in *A. hebraeum* ticks. *A. variegatum* ticks in the field have been found to have low infection rate (Camus, 1987), contrary to the findings of (Waghela

et al., 1991) who found artificially infected laboratory *A. variegatum* ticks acquired high infection rates close to fifty percent, and artificially infected laboratory *A. hebraeum* ticks acquired infection rates of over 90%, (Yunker *et al., 1*993). *A. variegatum* ticks used in this study were recently established in the laboratory (less than five generations) so they were not far removed from field ticks. Ticks in this study engorged during the prefebrile period or early in the febrile period whereas in studies by Waghela and colleagues, (1991 and Yunker and colleagues, (1993), ticks fed for a considerable part of the febrile period. *Cowdria* strains used in this study were of low virulence whereas the Kiswani used by Waghela *et al.,* (1991) is a more virulent strain and Crystal springs isolate, (Mahan *et al.,* 1992), was used to infect a vector that has been shown to be relatively susceptible to a broad spectrum of *Cowdria* strains infection (Mahan *et al., 1*995). Thus it would appear that while *A. variegatum* is less susceptible to *Cowdria* infection than *A. hebraeum*, (Mahan *et al., 1*995), *A. variegatum* may be even less susceptible to infection with low virulent strains of *Cowdria* such as the Bamba. In the work by Waghela *et al.,* (1991), ticks fed as larvae and assessed as nymphs appeared to have picked up a higher infection rate than ticks fed as nymphs and assessed as adults.

PCR has high sensitivity and can theoretically amplify a single DNA molecule (Innis and Gelfand, 1990). The pCS20 DNA probe is sensitive and specific for *Cowdria* DNA (Waghela *et al.,* 1991; Mahan *et al.,* 1992; Yunker *et al.,* 1993). It follows that primers derived from it ought to be sensitive and specific for *Cowdria* DNA also. Levels as low as 5.75 fg (Watson, 1993), of purified *Cowdria* DNA have been detected. Nested PCR is able to amplify DNA where a direct PCR has failed (Nicoll, 1994), and silver staining is much more sensitive than agarose gel (Somerville and Wang, 1981). Confirmation of specific nucleotide sequences can be performed by hybridisation with a labelled probe (Saiki *et al.,* 1988; Peter *et al.,* 1995). In this work only agarose gel was used but it was possible to detect 10 fg.

The use of *A. variegatum* primers was considered because amplification of *A. variegatum* would provide an 'internal quality control' on each amplification. There was apparent inability of the AB128/129 primers to amplify the two isolates

satisfactorily suggesting there are differences at the primer site in the nucleotide sequences of the two isolates which prevented proper recognition/annealing and hence amplification. The highly variable region named HE1 on the 16S rRNA gene (Anderson *et al.*, 1992), is the source of all HE primers. PCR test based on the 16S rRNA gene have successfully differentiated biovars of *Ureaplasma urealyticum* (Robertson *et al.*, 1993). However a small change in the amplification efficiency results in a dramatic change in total product yield (Xu and Larzul, 1991). The HE1(cr)/HE3(s) primers appeared to have a different detection ability for Asembo and Bamba DNA, (Figure 7.9) with or without the help from extra templates made by EC primers (Figures 7.10 & 7.11). The stability of the hybridisation complex, largely determines the specificity of the PCR (Innis and Gelfand, 1990). Where heterogeneity of pathogen is expected lower annealing temperature through a mismatched base pair between oligonucleotide and target decreases the melting temperature by 5°C and could prevent the primer from annealing and amplifying the target (Watson, 1993). In this work reducing the annealing temperature was found to improve the ability of the HE primers to amplify the different isolates. Also because starting DNA was suspected to be low (50 or less copies), a high number of cycles (45 cycles) were used with both AB and HE primers.

It can thus be concluded that the AB128/129 primers were more sensitive to Bamba DNA and HE1(cow)/HE3(s) in PCR was more sensitive to detection of Asembo DNA. The products of HE2/HE3(s) or HE2/HE3(l) were considered to be non specific to *Cowdria* although they did indicate that two tick samples contained an agent of the *Ehrlichiae* family. This may have been *Ehrlichia* or *Cowdria* which the specific primers may or may not have been able to amplify (Figure 7.7). The Bamba isolate was a *Cowdria* as intimated by clinical and PM findings and also presence of colonies in the endothelial cells of the brain by the method of Synge (1978) (not shown).

This work has shown how *Cowdria* is able to infect *Amblyomma* ticks before and during fever as has been demonstrated before, (Barré and Camus, 1987; Waghela *et al.*, 1991). In contrast to the findings of Mahan *et al.*, (1992), where the pCS20 probe

(the source of AB primers) detected eight *Cowdria* strains from different regions of Africa and the Caribbean, it was apparent that the different isolates have differences among them at the two sites used to derive the primers. Conditions needed to be made less stringent to increase the annealing stability of the primer/ DNA complex. Findings in this study underscores the need to assess the classification of *Cowdria* as many strains have been found with diverse characteristics from each other, some being more like *Ehrlichia* even at the molecular level. With the report of an *Ehrlichia* having been experimentally transmitted by an *Amblyomma*, (Ewing *et al., 1995*), the question arises as to the demarcation between the two agents. Whether *Cowdria* can be transmitted by vectors other than *Amblyomma* and whether *Ehrlichia* like organisms can transform into *Cowdria* like organisms, (Du Plessis, 1990; 1993) is unknown.

7.6 CONCLUSION

It was not possible to draw any conclusions about tick infection rates between the two isolates for the two species of ticks. Perhaps this is because mild strains result in low infection rates in ticks, or the kinetics of infection with mild strains in sheep is quite different from virulent strains and the ticks were applied at relatively early stage. PCR methods for Bamba and Asembo isolates were developed and a method which detects a wide range of Kenyan *Cowdria* stocks could be applied to others.

Table 7.1: Tick numbers of the *A. variegatum* and *A. gemma* ticks selected from one sheep each infected with the Asembo and Bamba *Cowdria* isolates.

Date	Av-patch no 1 (numbers)	Ag patch no 2 (numbers)	Av patch no 3 (numbers)	Ag patch no 4 (numbers)	Total
Sheep 177 (Asembo)					
9.4.96	10(101-110)	10(111-120)			
10.4.96	10(121-130)	10(131-140)	7(205-211)	7(226-232)	
11.4.96	10(141-150)	10(175-184)	10(165-174)	10(236-245)	
12.4.96	6(159-164)	6(194-200)	10(185-194)	10(246-255)	
13.4.96			2(214-215)	2(256, 260)	130
Sheep 183 (Bamba)	7(261-267)	7(283-289)			
13.4.96	10(268-277)	10(293-302)			
14.4.96	2(278-279)	2(303, 312)			
15.4.96			7(330-336)	7(357-363)	
16.4.96			8(337)344	8(367-374)	
17.4.96			1(345)	1(382)	
18.4.96			1(346)	1(392)	72
19.4.96					
Sheep 104 (clean)					
15.11.95	6(91-96)				6
Sheep 8 (clean)					
15.11.95	4(97-100)				4
Total	**65**	**55**	**46**	**46**	**212**

Table 7.2 DNA primers their sequence and theoretical amplification specificity

Primer code	Sequence	Theoretical primer specificity
	pCS20 derived primer set from 5′-3′	
AB 128	ACTAGTAGAAATTGCACAATCTAT	*C. ruminantium*, (Crystal Spring), (Mahan et al, 1992)
AB 129	TGATAACTTGGTGCGGGAAATCCTT	*C. ruminantium*, (Crystal Spring)
forward	HE primers derived from the 16S rDNA sequences from 5′-3′	
HE1(cr)	CAGTTATTTATAGCTTCGGCTATGAG	*C. ruminantium* (Crystal Spring), (Dame, *et al.*, 1992; Van Vliet *et al.*, 1992)
HE1(cow)	CAGTTATTTATAGCTTCGGCTATRAGTATCTG	*C. ruminantium*, (Senegal, Crystal Spring) (Sumption, K., unpublished result)
HE2	GTGGCAGACGGGTGAGTAATGC	*E. canis, E. phagocytophila, E. chaffeensis, E equi, E. ewingi, E. risticii, C. ruminantium* and *Anaplasma marginale*
reverse		
HE3(s)	GGTACCGTCATTATCTTCCC	*E. canis, E. phagocytophila, E. chaffeensis, E. equi, E. ewingi, E. risticii, C. ruminantium* and *Anaplasma marginale*
HE3(l)	CTATAGGTACCGTCATTATCTTCCC	*C. ruminantium*, (Senegal, Crystal Spring), *E. canis, E. chaffeensis, E. ewingii*
	EC primers derived from the 16S rRNA gene	
EC11	AAGGATCCGGACTACHAGGGTATCTAAT	universal bacteria, (Anderson, *et al.*, 1991)
EC12	AATCTAGAGTTTGATCMTGG	universal bacteria, (Anderson, *et al.*, 1991)
	Avar primers derived from the 16S rRNA gene (of ticks)	
Avar-F1	TAAGGACAAGAAGACCCTAAGAAT	*Amblyomma variegatum*, (Sumption, K., unpublished result)
Avar-R2	TAACTTCTTCATTAAATAAGAATCC	*Amblyomma variegatum*, (Sumption, K., unpublished result)

KEY

R = G or A H = C, T, or A M = A or C

Table 7.3: Primer combination and PCR amplification cycle conditions

Programme	Primers	No. of cycles	Denaturation	Annealing	Extension	Final extension for 10 minutes	Hold
A	AB128/AB129 Avar-F1/Avar-R2	45	94°C, 1 min	55°C, 1 min	72°C, 2 min	72°C	22°C
B	HE1(cr)/HE3(l), HE2/HE3(l)	30	94°C, 1 min	60°C, 1 min	72°C, 1.5 min	72°C	22°C
C	HE1(cow)/HE3(l) HE2/HE3(s) HE1(cow)/HE3(s)	45	94°C, 1 min	52°C, 1 min	72°C, 1.5 min	72°C	22°C
D	HE1(cow)/HE3(l) HE2/HE3(s)	45	94°C, 30 sec	60°C,30 sec	72°C, 30 sec	72°C	22°C
E	EC11/EC12	10	88°C, 1 min	55°C, 2 min	70°C, 1.5 min	70°C	22°C
F	EC11/EC12	37	88°C, 1 min	45°C, 2 min	70°C, 1.5 min	70°C	22°C
G	HE1(cow)/HE3(s;	30	94°C, 1 min	52°C, 1 min	72°C, 1 minute, inc. 1 sec cycle	72°C	22°C
H	HE1(cow)/HE3(s)	45	94°C, 1 min	52°C, 1 min	72°C, 1 min inc. 1 sec per cycle	72°C	22°C

Pre-amplification protocol

1) hot start 90°C - cycler preheated to 90°C before samples are placed
2) samples denatured pre mix by 96°C for 10 minutes (before addition of master mix)
3) samples denatured post mix by one cycle of 95°C for 4 minutes, 60°C for 1 minute annealing and 72°C for 1 minute extension
4) samples denatured post mix by one cycle of 95°C for 4 minutes, 60°C 1 minute for annealing and 70°C 1 minute for extension

Table 7.4: DNA concentration in ticks extracted from different tick species fed of different sheep

Sheep #	Median	Range	Median	Range
	A. variegatum		*A. gemma*	
104	1395	725-1680	none	na
8 (control)	1495	710-1825	none	na
177	1197.5	840-1720	1186.25	445-2085
(Asembo)	1380	790-2131	1397.5	580-2654
183	1252.5	800-1742.5	1260	1015-2030
(Bamba)	1337.5	755-2020	1420	1080-2125

KEY

The values obtained by measuring 10 µl aliquots have been preferred as more accurate.

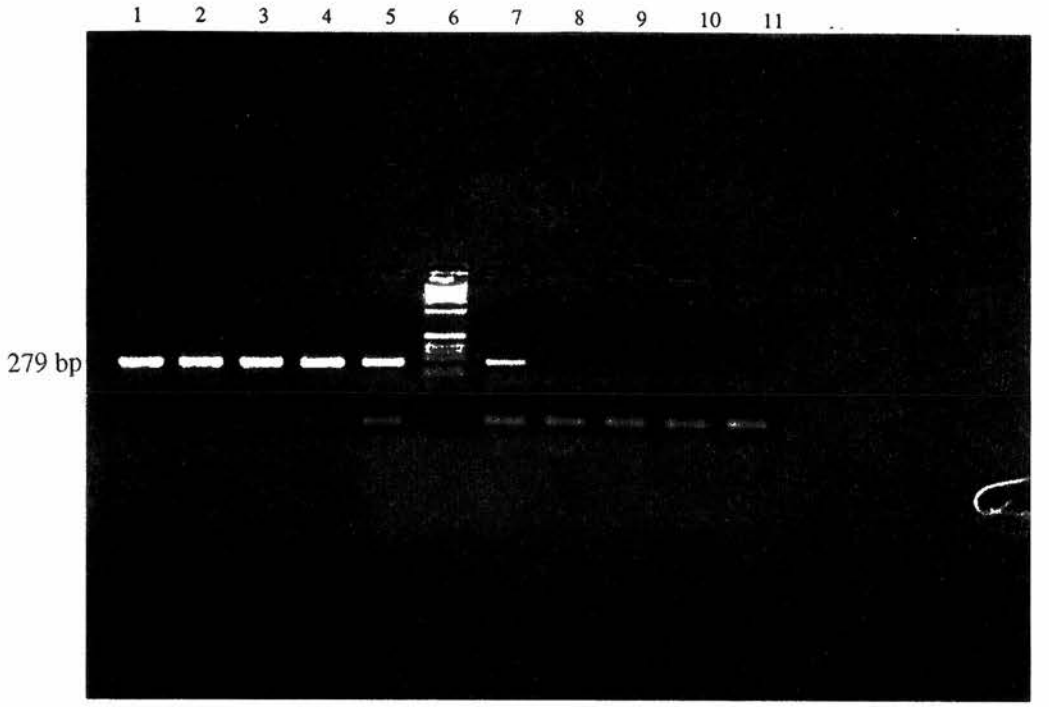
Table 7.5 Tick infection rates

Sheep #	Patch 1	Patch 2	Patch 3	Patch 4
	A. variegatum	*A. gemma*	*A. variegatum*	*A. gemma*
104	0/6*	na	na	na
8 (control)	0/4	na	na	na
177	0/36	0/36	0/29	0/29
(Asembo)				
183	0/17	1/19	1/17	0/19
(Bamba)				

KEY

0/6* - zero out of six *A. variegatum* ticks applied on sheep no. 104 were positive for *Cowdria ruminantium* infection

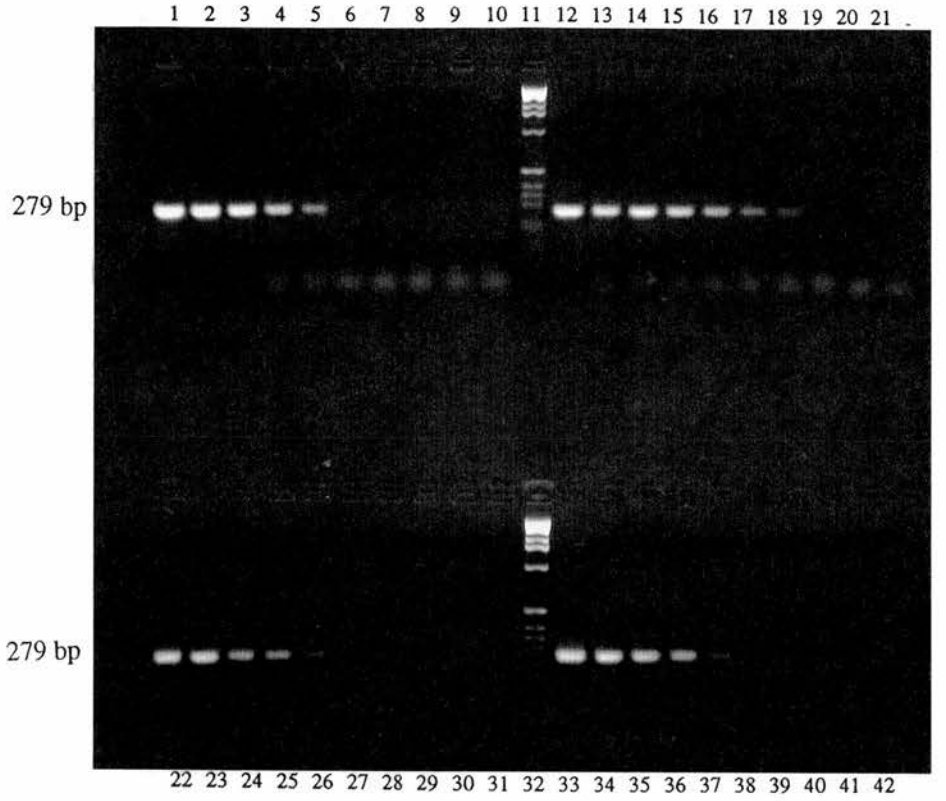
na - not applicable



KEY

Lane	Sample	Result	Lane	Sample	Result
1	10 ng/µl	+	7	100 fg/µl	+
2	1 ng/µl	+	8	10 fg/µl	+
3	0.1 ng/µl	+	9	1 fg/µl	-
4	10 pg/µl	+	10	tick pool	-
5	1 pg/µl	+	11	MQ H2O	-
6	MWt	Kb ladder			

Figure 7.1 To establish the sensitivity of AB128/AB129 primers for control *C ruminantium* (Welgevonden) DNA



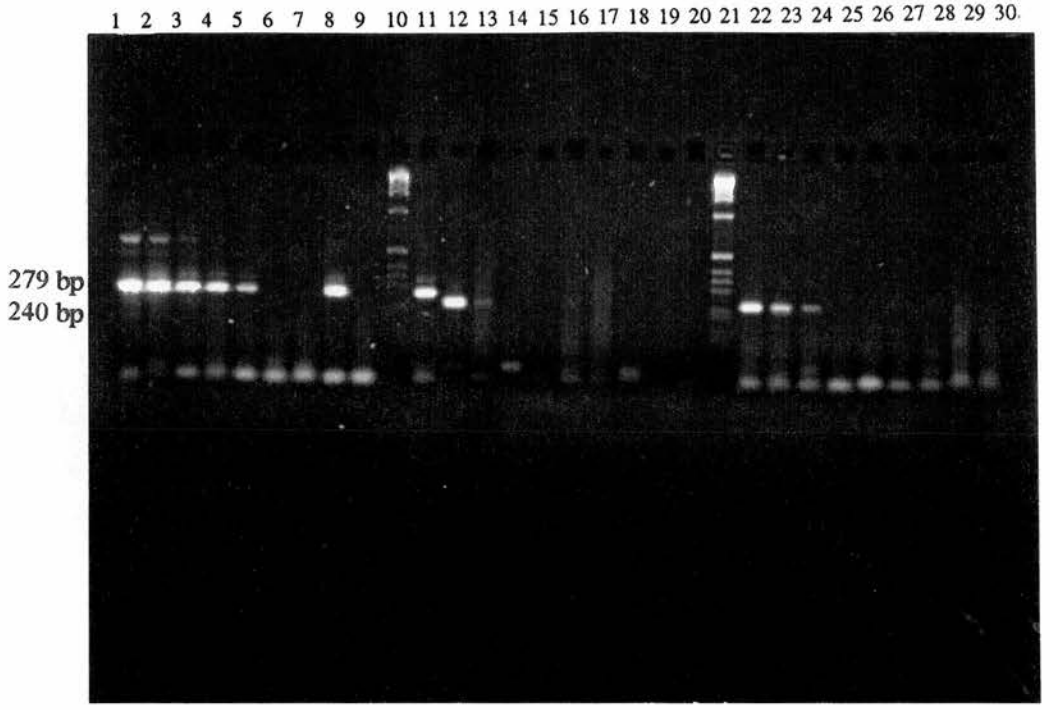
KEY

Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result
1	10 ng/µl	+	12	10 ng/µl	+	22	10 ng/µl	+	32	Kb ladder	
2	1 ng/µl	+	13	1 ng/µl	+	23	1 ng/µl	+	33	10 ng/µl	+
3	0.1 ng/µl	+	14	0.1 ng/µl	+	24	0.1 ng/µl	+	34	1 ng/µl	+
4	10 pg/µl	+	15	10 pg/µl	+	25	10 pg/µl	+	35	0.1 ng/µl	+
5	1 pg/µl	+	16	1 pg/µl	+	26	1 pg/µl	+	36	10 pg/µl	+
6	100 fg/µl	-	17	100 fg/µl	+	27	100 fg/µl	-	37	1 pg/µl	+
7	10 fg/µl	-	18	10 fg/µl	+	28	10 fg/µl	-	38	100 fg/µl	-
8	1 fg/µl	-	19	1 fg/µl	-	29	1 fg/µl	-	39	10 fg/µl	-
9	tick pool	-	20	tick pool	-	30	tick pool	-	40	1 fg/µl	-
10	H2O	-	21	H2O	-	31	H2O	-	41	tick pool	-
11	Kb	ladder							42	H2O	-

KEY

Four test sample sets of Welgevonden TC EBs dilutions as shown above. Constant tick pool DNA was added in the first three sets (lane nos. 1-8, 12-19 & 22-29).The fourth set, no tick DNA added (lane nos. in 33-40). $MgCl_2$ concentration made to 3 µmolar in first and fourth sets, 4 µmolar and 5 µmolar in second and third sets respectively.

Figure 7.2: The sensitivity of AB128/AB129 primers to amplify control DNA dilutions in three concentrations of magnesium chloride and in the presence and absence of tick pool extract

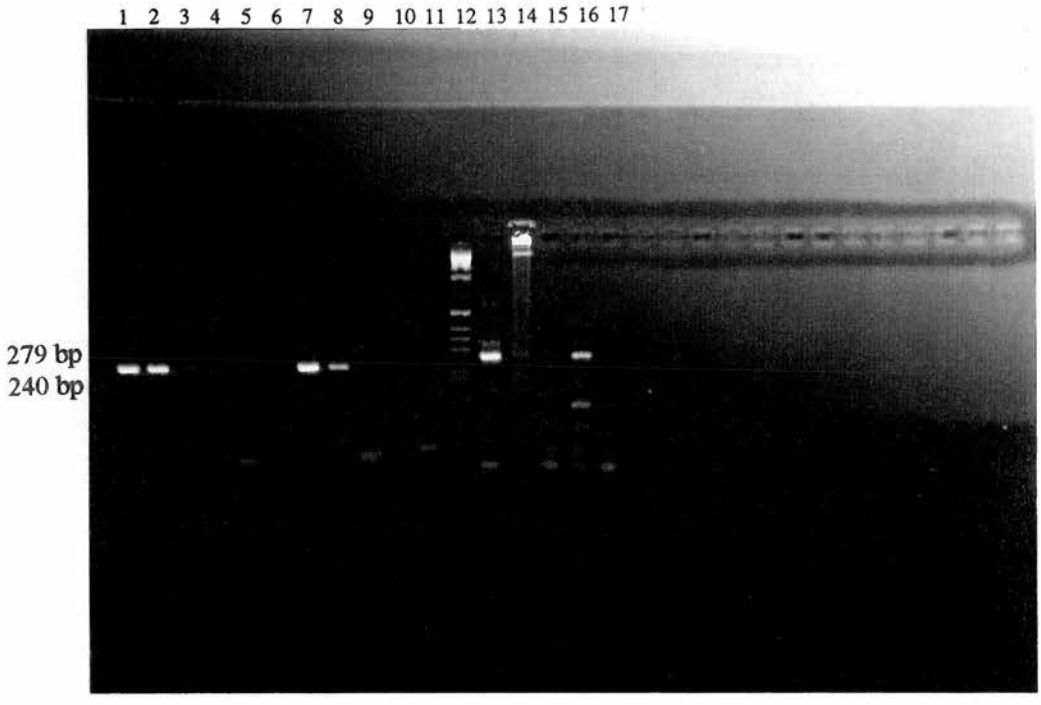


KEY

Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result
1	1 ng/µl	+	11	TC EBs	+	21	Kb ladder	
2	0.1 ng/µl	+	12	tick 10^{-1}	+	22	tick 10^{-1}	+
3	10 pg/µl	+	13	tick 10^{-2}	(+)-	23	tick 10^{-2}	+
4	1 pg/µl	+	14	tick 10^{-3}	-	24	tick 10^{-3}	+
5	100 fg/µl	+	15	tick 10^{-4}	-	25	tick 10^{-4}	-
6	10 fg/µl	-	16	tick 10^{-5}	-	26	tick 10^{-5}	-
7	1 fg/µl	-	17	tick 10^{-6}	-	27	tick 10^{-6}	-
8	0.1 ng/µl	+	18	tick 10^{-7}	-	28	tick 10^{-7}	-
9	H2O	-	19	tick 10^{-8}	-	29	tick 10^{-8}	-
10	Kb ladder		20	H2O		30	H2O	-

These are three test reaction sets: first set (lane sample nos. 1-8), Welgevonden TC EBs dilutions as shown, second and third sets (lane sample nos. 12-28) tick pool dilutions as shown. Controls sterile MilliQ water in lane 9, 19 and 29. Both sets of primers (AB128/AB129 and Avar-F1/Avar-R2) put in sets one and three. Set two (lane sample nos. 12-18) added only Avar -F1/Avar-R2 primers. One sample, lane no 11 positive control (10 pg/µl) Welgevonden EBs dilution containing only AB128/AB129 primers.

Figure 7.3: The ability of AB128/AB129 primers to amplify control *Cowdria* DNA dilutions in the presence of Avar-F1/Avar-R2 primers and Avar-F1/Avar-R2 primers to amplify tick pool DNA in three presence and absence of AB128/AB129 primers.

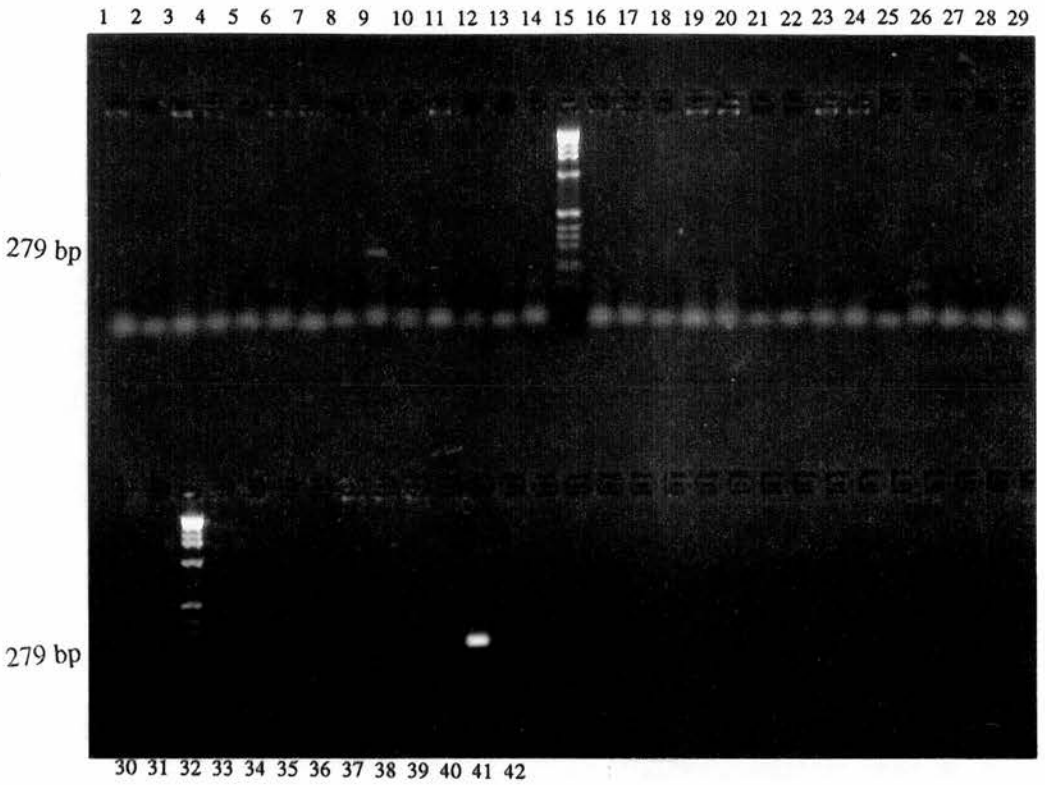


KEY

Lane	Sample	Result	Lane	Sample	Result
1	tick pool 10-1	+	10	tick pool 10-4	-
2	tick pool 10-2	+	11	tick pool 10-5	-
3	tick pool 10-3	(+)	12	MWt marker	Kb ladder
4	tick pool 10-4	-	13	TC bc 10 pg/µl	+
5	tick pool 10-5	-	14	PCR prod.	(+)
6	H2O	-	15	Asembo(Q)	-
7	tick pool 10-1	+	16	Bamba(Q)	+
8	tick pool 10-2	+	17	H2O	-
9	tick pool 10-3	-			

Tick pool DNA dilutions as shown amplified with the tick DNA primers Avar-F1/Avar-R2, and Welgevonden TC EBs (+ve control), Qiagen extracted (Q) Asembo and Bamba DNA samples (to determine the amplifiability of Asembo and Bamba DNA) reacted using the AB128/AB129 primers.

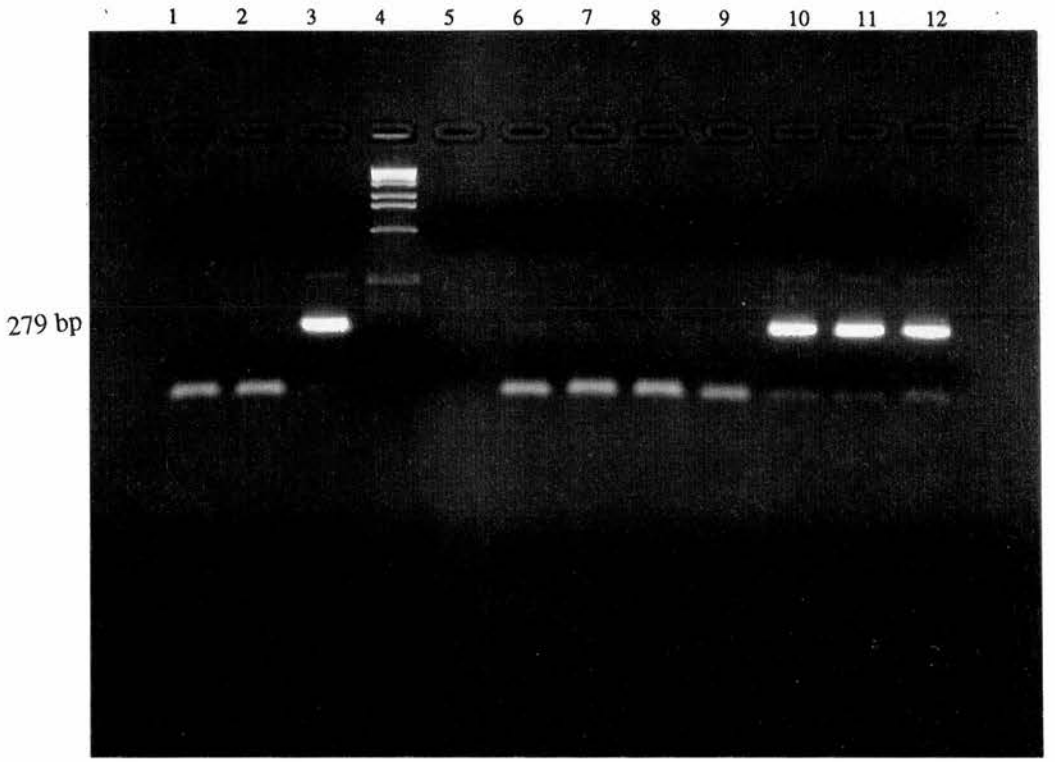
Figure 7.4: To determine the ability of Avar-F1/Avar-R2 primers to amplify tick pool DNA and AB128/AB129 primers to amplify Qiagen extracted Bamba and Asembo *Cowdria* DNA.



KEY

Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result
1	261	-	12	287	-	22	274	-	33	299	-
2	262	-	13	288	-	23	275	-	34	300	-
3	263	-	14	289	-	24	276	-	35	301	-
4	264	-	15	Kb ladder		25	277	-	36	302	-
5	265	-	16	268	-	26	293	-	37	278	-
6	266	-	17	269	-	27	294	-	38	279	-
7	267	-	18	270	-	28	295	-	39	311	-
8	283	-	19	271	-	29	296	-	40	312	-
9	284	+	20	272	-	30	297	-	41	TC EBs	+
10	285	-	21	273	-	31	298	-	42	MQ H2O	-
11	286	-				32	Kb ladder				

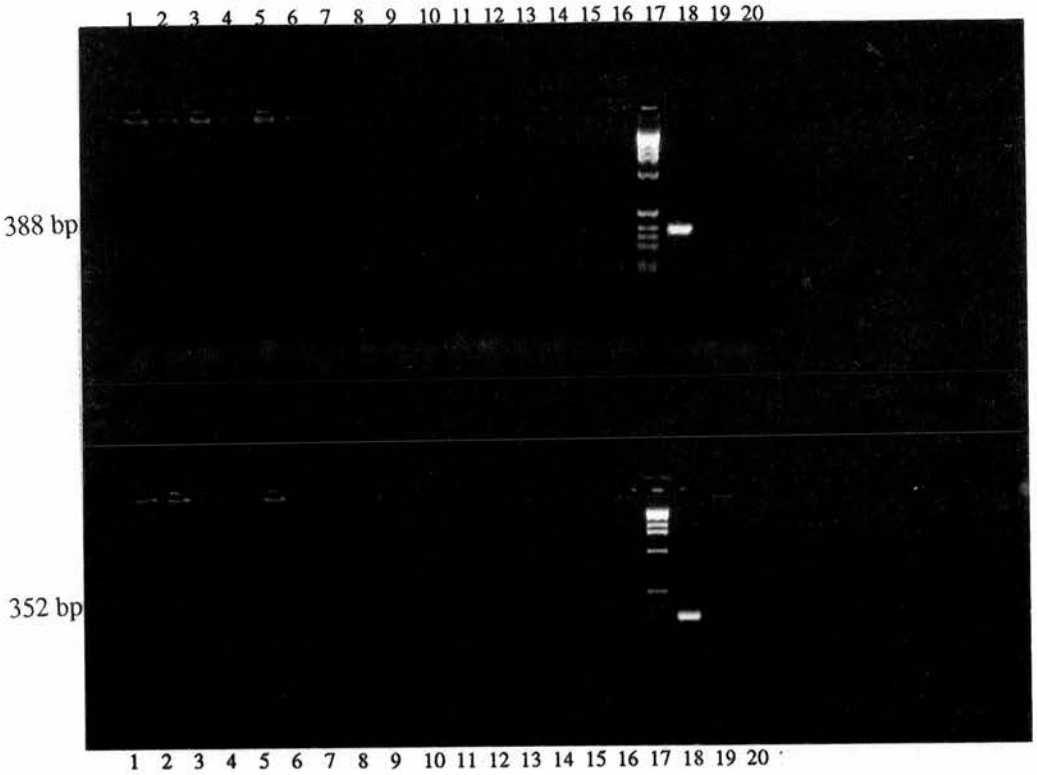
Figure 7.5: Amplification of DNA from tick extracts of *C. ruminantium* (Bamba) by AB128/AB129 primers in 4μM $MgCl_2$



KEY

Lane	Sample	Result	Lane	Sample	Result
1	MQ H2O	-	7	248 (Bamba)	-
2	tick pool	-	8	340 (1/10)(Bamba)	-
3	TC EBs	+	9	340 (1/100)(Bamba)	-
MWt	Kb ladder		10	Kathiani 972	+
5	Senegal	-	11	Isiolo	+
6	Asembo	(+)	12	Baragoi	+

Figure 7.6: The sensitivity of AB128/AB129 primers for different *C. ruminantium* isolates.



KEY

Lane	Sample #	Results HE1/HE3	Results HE2/HE3	Lane	Sample	Results HE1/HE3	Results HE2/HE3
1	101	-	-	11	115	-	-
2	102	-	-	12	116	-	-
3	103	-	-	13	284	-	-
4	104	-	-	14	340	-	-
5	105	-	-	15	Asembo	-	(+)
6	106	-	-	16	Bamba	-	(+)
7	111	-	-	17	Kb ladder		
8	112	-	-	18	TC EBs	+	+
9	113	-	-	19	tick pool	-	-
10	114	-	-	20	MQ H2O	-	-

HE1 = HE1(cr) HE3 = HE3(l)

Top gel half, primers HE1(cr)/HE3(l), bottom gel half, primers HE2/HE3(l) samples duplicates of top gel half.

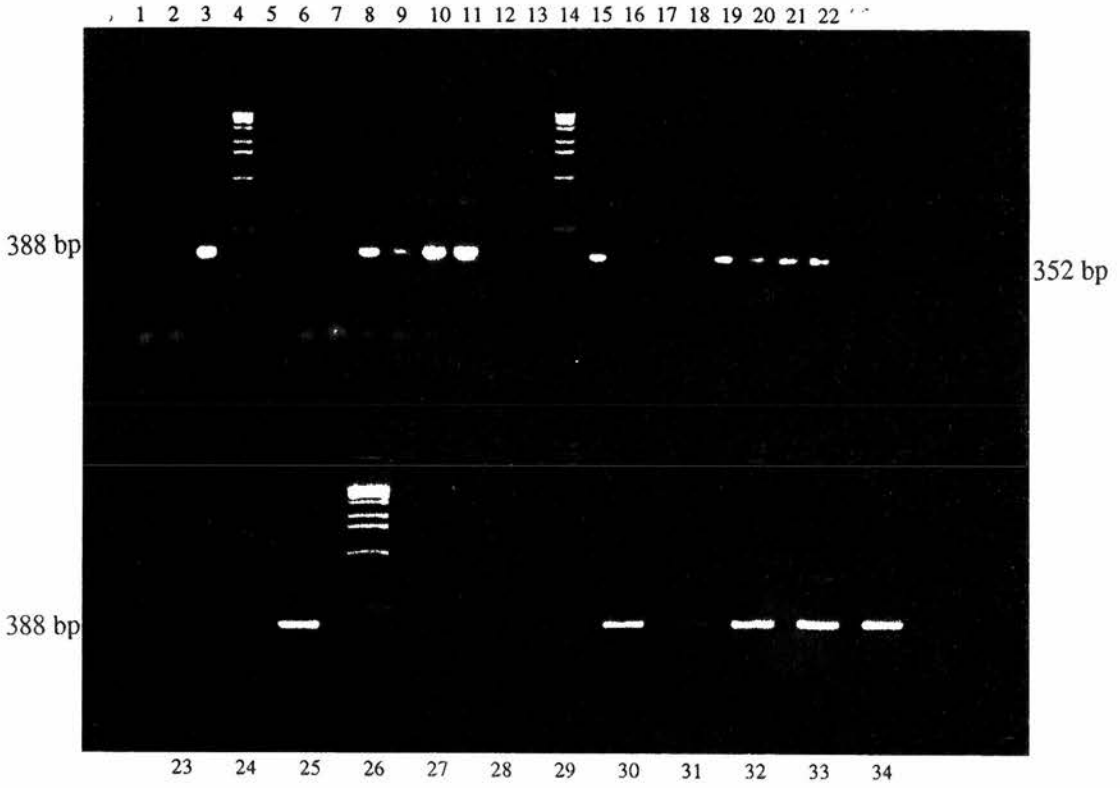
Figure 7.7: The sensitivity of HE1(cr)/HE3(s), and HE2/HE3(s) primers to Asembo tick extracts and Qiagen extracted Asembo and Bamba DNA under standard annealing temperature.

KEY

Lane	Sample	Result	Lane	Sample	Result
1	MQ H2O	-	8	Asembo	-
2	tick pool	-	9	Kathiani 972	-
3	TC EBs	+	10	Isiolo	-
4	284	-	MWt	Kb ladder	
5	340	-	12	H2O	-
6	Asembo	-	13	tick pool	-
7	Bamba	-	14	Asembo	+

First set (Lane samples 1-10), HE1(cow)/HE3(l) primers. Second set (samples 12-14), HE2/HE3(s) primers.

Figure 7.8: Amplification of DNA from different *C. ruminantium* isolates by HE1(cow)/ HE3(l) primers and Asembo DNA by HE2/HE3(s) at the standard annealing temperature.

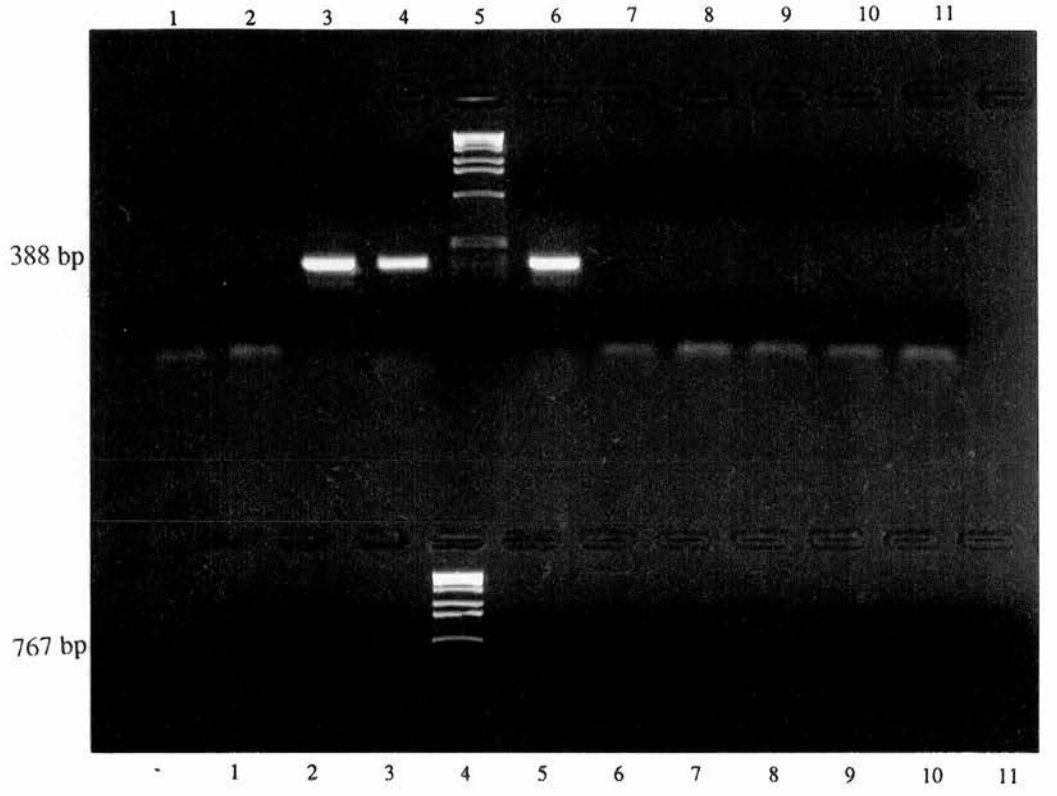


KEY

Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result
1	MQ H2O	-	10	Kathiani 972	+	19	Asembo	+	28	284	-
2	tick pool	-	11	Isiolo	+	20	Bamba	+	29	340	-
3	TC EBs	+	12	MQ H2O	-	21	Kathiani 972	+	30	Asembo	+
4	Kb ladder		13	tick pool	-	22	Isiolo	+	31	Bamba	(+)
5	Senegal	-	14	Kb ladder		23	MQ H2O	-	32	Kathiani 972	+
6	284	-	15	TC EBs	+	24	tick pool	-	33	Isiolo	+
7	340	-	16	Senegal	-	25	TC EBs	+	34	Baragoi	+
8	Asembo	+	17	284	(+)	26	Kb ladder				
9	Bamba	(+)	18	340	(+)	27	Senegal	-			

Three sets of sample tests each with a different HE primers combination, viz.: first set samples (lane nos. 1-11), HE1(cow)/HE3(l) primers. Second set samples (lane nos. 12-22), HE2/HE3(s) primers. First and second sets on top gel half. Third set (lane nos. 23-34) HE1(cow)/HE3(s) primers, (lower gel half).

Figure 7.9: Amplification of DNA from different *C. ruminantium* isolates by HE1(cow)/HE3(l), HE2/HE3(s) and HE1(cow)/HE3(s) primers at a reduced annealing temperature.

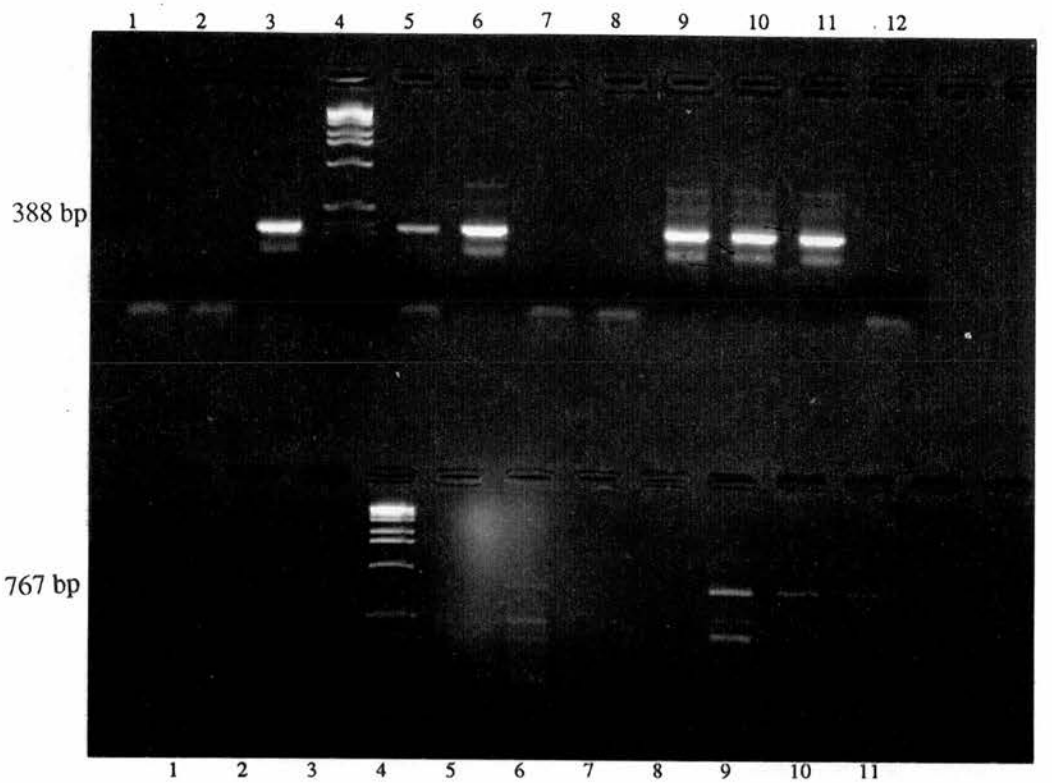


KEY

Lane	Sample	Result	Lane	Sample	Result
1	H2O	- -	7	H2O	- -
2	tick	- -	8	248 (Bamba)	- -
3	TC EBs	- +	9	340 (Bamba)	- -
4	Senegal	- +	10	340 (1/10) (Bamba)	- -
MWt	Kb ladder		11	340 (1/100) (Bamba)	- -
6	Asembo	- +			

First phase EC11/EC12 primers, (lower gel half), viz.: Second phase, HE1(cow)/HE3(s), (top gel half),

Figure 7.10: **Amplification of DNA from different *C. ruminantium* isolates with HE1(cow)/HE3(s) primers in a nested PCR with EC11/EC12 primers**

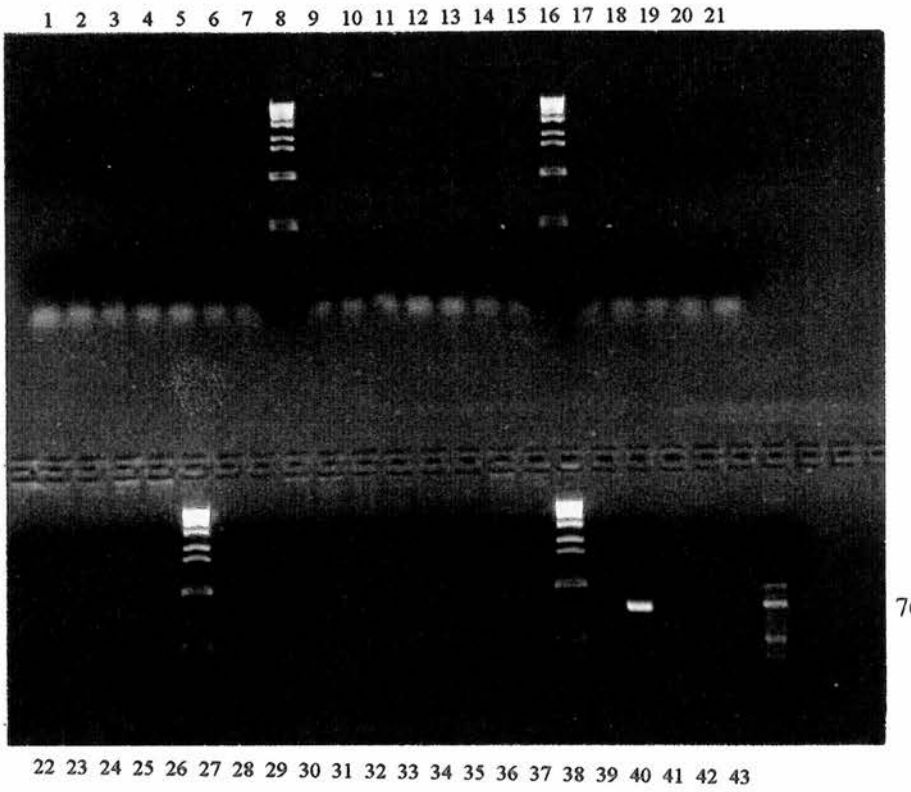


KEY

Lane	Sample	Result	Lane	Sample	Result
1	H2O	- -	7	248 (Bamba)	- -
2	tick pool	- -	8	340 (Bamba)	- -
3	TC EBs	(+)+ve con	9	Kathiani 972	(+) +
MWt	Kb ladder		10	Isiolo	(+) +
5	Senegal	- +	11	Baragoi	(+) +
6	Asembo	(+)+	12	H2O	-

First phase EC11/EC12 primers (lower gel half) Phase two HE1(cow)/HE3(s) (upper gel half),

Figure 7.11: The ability of nested PCR to produce a product when EC11/EC12 primers perform the complete PCR (for those primers), followed by PCR with HE1(cow)/HE3(s) primers.

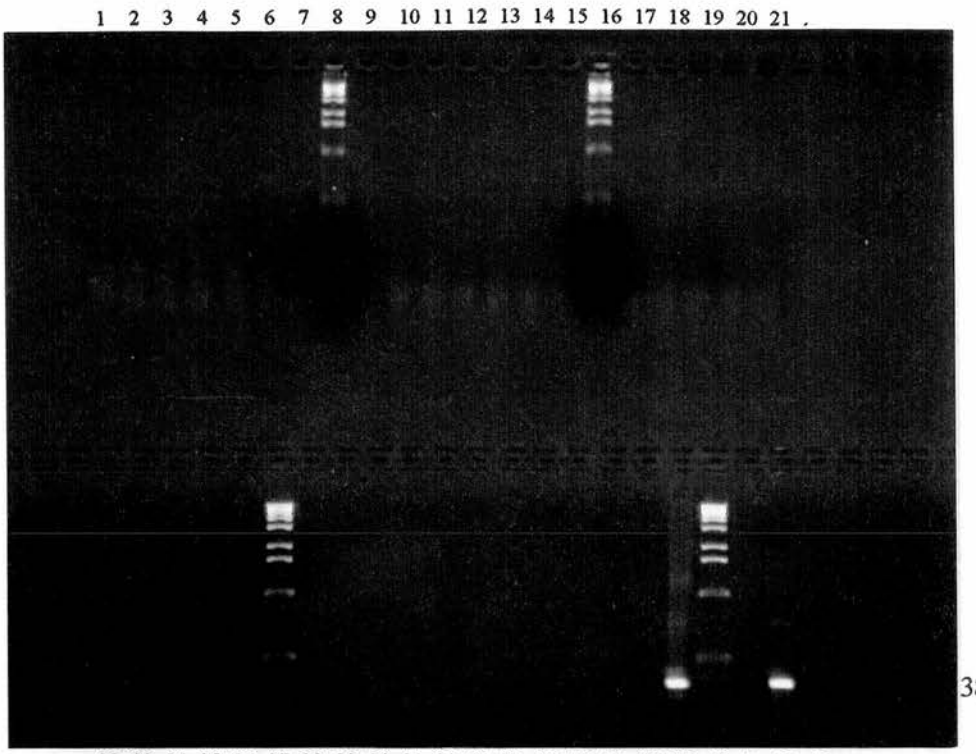


Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result
1	205	-	12	229	-	23	171	-	34	242	-
2	206	-	13	230	-	24	172	-	35	243	-
3	207	-	14	231	-	25	173	-	36	244	-
4	208	-	15	232	-	26	174	-	37	245	-
5	209	-	16	Kb ladder		27	Kb ladder		38	Kb ladder	
6	210	-	17	165	-	28	236	-	39	. H2O	-
7	211	-	18	166	-	29	237	-	40	+ve con*	+
8	Kb ladder		19	167	-	30	238	-	41	tick*	-
9	226	-	20	168	-	31	239	-	42	Asembo(q)	(+)
10	227	-	21	169	-	32	240	-	43	H2O	-
11	228	-	22	170	-	33	241	-			

Key

* empty well after this sample

Figure 7.12a: Amplification of Asembo tick extracts by nested PCR of EC11/EC12 primers with HE1(cow)/HE3(s) primers (First gel: first phase EC11/EC12 primers)



KEY

Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result	Lane	Sample	Result
1	205	-	12	229	-	23	171	-	34	242	-
2	206	-	13	230	-	24	172	-	35	243	-
3	207	-	14	231	-	25	173	-	36	244	-
4	208	-	15	232	-	26	174	-	37	245	-
5	209	-	16	Kb ladder		27	Kb ladder		38	H2O	-
6	210	-	17	165	-	28	236	-	39	+ve con.	+
7	211	-	18	166	-	29	237	-	40	Kb ladder	
8	Kb ladder		19	167	-	30	238	-	41	tick	-
9	226	-	20	168	-	31	239	-	42	Asembo(q)	(+)
10	227	-	21	169	-	32	240	-	43	H2O	-
11	228	-	22	170	-	33	241	-			

(second phase, second gel, HE1(cow)/HE3(s) primers

Figure 7.12b: Amplification of Asembo tick extracts by nested PCR of EC11/EC12 primers with HE1(cow)/HE3(s) primers

GENERAL DISCUSSION

At the start of these studies there was only one available Kenyan *Cowdria* isolate, the Kiswani isolate. This was principally because heartwater is largely endemic in this country and with endemic stability prevailing it has been relegated to low priority. However, it was also because isolation of *Cowdria* is a laborious process requiring much patience and luck (FAO, 1984; Ilemobade and Blotkamp, 1978). The isolation of *Cowdria* was central to the present study.

All isolates were obtained from *Amblyomma* species in eight districts where livestock (cattle, sheep and goats) co-exist with wildlife on the same pastures. Heartwater is undoutably also present and endemic in parts of the country where isolation was not attempted and where *Amblyomma* occurs. A number of isolates were obtained from *A. gemma* and *A. lepidum,* 'the lesser vectors'. The majority of the isolates were obtained from *A. variegatum,* which is the most widespread vector in the country and probably it is the most important vector of heartwater in Kenya. Most of the ticks were collected from cattle, a few were from small stock, but some camels were also donors of some of the *Amblyomma* ticks at one (Isiolo) isolation site. Overall, the epidemiology of heartwater in this country is likely to be a complex involving several *Amblyomma* vectors, wildlife and domestic livestock (Petney *et al., 1*987).

Lack of infection (no seroconvertion) following sub-inoculation of blood from carrier animals into susceptible sheep demonstrated the paucity of the heartwater agent in recovered animals. These sheep reacted with a moderate fever, but were found to be harbouring *Anaplasma ovis,* previously reported by Neitz, (1939). However, *Cowdria* was isolated from ticks in some of these areas. *Amblyomma* are consumers of large amounts of blood and are capable of concentrating it many fold in their meal (Norval *et al., 1*992). They are therefore able to amplify infection from animals where it is undetectable except perhaps by means of PCR (Kock *et al., 1*995). Although *Ehrlichia* cross reacts serologically with *Cowdria (*Logan *et al., 1*987; Jongejan *et al., 1*991a), most animals at isolation sites can be suspected of having been exposed to *Cowdria* because isolates were made, and between 75% -100% of cattle had high IL antibody to *Cowdria* antigen. The low infection rates obtained by feeding ticks on an infected animal in this study (although the timing fell slightly short) would suggest similar low infection rates occur in the field. Ticks have been declared positive on PCR results, from which no isolate was made (Wesonga *et al., 1*993). As pointed out before

(Camus and Barré, 1992), apparently infected ticks may fail to transmit heartwater. This might be due to coordination between pathogen development and arthropod host activities (Munderloh and Kurtii, 1995), restricting some of the organisms in a particular instar from being transmitted. During these studies there were regions where high antibody titres of the donor cattle suggested that animals had very likely been exposed to *Cowdria* and yet no isolate was made e.g. Kangundo. This would support there being a low infection rate in this and many *Amblyomma* tick populations in the field. It was surprising therefore that isolation was possible from half (8 out of 16) of the areas where isolation was attempted. It may be that the agent is not in a transmissible stage or in an activated state when transmission fails to take place and this observation should be investigated further. However not all ticks had low infection rates because as few as 2 to 6 attached ticks resulted in three *Cowdria* isolates. The expectation is that there is variable infection rates in different areas and species of ticks (Du Plessis and Malan, 1987; Camus, 1987 quoted by Camus *et al.*, 1996; Norval *et al., 1990*; Gueye *et al., 1993*). This points to a situation of pockets of endemic areas in Kenya, each probably having exclusive populations and events possibly not affecting the other 'pockets'.

The evidence in this study points to populations of *Cowdria* having distinct antigenic and immunogenic characteristics. The uniqueness of the isolates was seen in the character of infection in sheep and mice (Chapters 4 & 6) and cross protection patterns (chapter 5) with the different isolates. The spectrum seen in this study suggests that there may be similar areas of Kenya which differ in heartwater disease. It is interesting to note the similarities between certain Kenyan isolates and those obtained from other regions of Africa. For example the Asembo isolate was pathogenic to mice and sheep but not cattle (R. Rumberia pers. com. 1996), like the Kumm stock (Du Plessis and Kumm, (1971). The Kiswani isolate was latent in mice like the Ball 3 (Haig, 1952) and Senegal (Wassink and Jongejan, 1990) stocks while the Baragoi isolate was equally virulent to cattle sheep and mice like the Welgevonden stock (Du Plessis, 1985b).

These characteristics in different hosts, may be due to the antigenicity of the different agents but perhaps are more related to immunological responses they induce in the different hosts.

The outcome of cross-immunity trials is still bewildering because immunity to one isolate may not enhance to that induced by another and there is inequality of cross protection between any two isolates: one gives more or less protection against the other. From the regression of the reaction indices of the challenge isolates on the reaction indices of the immunising isolates (Figure 5.1b), it would appear that between *Cowdria* isolates there exist a possibility of four broad outcomes depending on whether each kills all untreated hosts (virulent) or spares some untreated hosts (low virulent): 1) a low virulence isolate protects against other low virulence isolates (low RI 1°/ low RI 2°); 2) a low virulence isolate does not protect against a high virulence isolate (low RI 1°/ high RI 2°); 3) a high virulence isolate protects against other high and low virulence isolates (high RI 1°/ low RI 2°) and 4) a high virulence isolate does not protect against high virulence isolates partially or totally (high RI 1°/ high RI 2°). This analysis is too clear cut as 'low' virulent isolates have a large range of relative virulence to one another. However, most high virulence isolates have been observed to protect against both low and high virulence isolates whereas, some low virulence isolates protect against highly virulent isolates but this appear to be the exception rather than the rule.

It would appear that relatedness of *Cowdria* isolates at the virulence antigens is important in inducing protective immunity against other isolates of similar or lesser virulence. The high virulence isolates were more related to each other (Nicoll *et al.* 1997) in this study and there was partial to complete cross protection without any deaths. Less related isolates (Du Plessis et al., 1989; Jongejan et al., 1988; 1991d) appear to cross protect less and with accompanying fatalities in a proportion of the animals. The studies on cross protection suggest that infection, virulence and cross protection are a function of the host species and the infecting agent. An infection sets up a response pattern that is repeated when the agent is next encountered (Mosmann and Coffman, 1989). The response to a different *Cowdria* must match the established pattern of immune responses (or perform close or superiorly) for the host to mount effective counter attack.

Cowdria gives us a unique model to study some of these immune responses in different hosts. For example in this study, mice developed an antibody but not disease with Bamba rickettsia, which did not protect them against the virulence of Asembo strain,

although it caused amelioration of some pathological lesions in the immunised mice. In contrast sheep immunised with the Bamba isolate were protected against Asembo virulence which was recognised as being a similar 'immunotype' in this species. The results suggest that in mice, like in sheep virulence was related to immunogenicity. In mice the Bamba isolate was very mild and did not protect against the virulence of Asembo whereas in sheep both isolates were relatively mild and cross protected sheep against each other. Overall, the strains that are virulent to more host species appear to be the ones that are cross protective against more isolates but they too may fail to cross protect against some isolates.

Post mortem diagnosis of heartwater has been assumed to be possible with well made smears soon after death. Colonies in the brain crush smears has been taken to be the gold standard for diagnosis and confirmation of *Cowdria.* From the evaluation of post mortem diagnosis and creating a diagnostic index in this study, it was apparent that colonies in the brain may not be a reliable way to confirm diagnosis even under ideal conditions for some *Cowdria* strains. There was a reduction of colonies in the endothelial cells of the brain as well as in neutrophils infections involving the 'mild' isolates. This leaves scientists still in need of a method to confirm the presence of the organism and the disease, mostly with the mild isolates but also with some virulent isolates. Several methods may need to be utilised in the difficult cases before an absolute diagnosis is pronounced. Since a high reaction index which was associated with improved diagnostic detection, can only be arrived at after the conclusion of the infection, diagnosis of deaths associated with high virulence may be easier but, one must persevere to make a confirmatory diagnosis or miss the diagnosis.

Fewer colonies in the brain endothelium, does not appear to be a more *Ehrlichia* like characteristic. *Ehrlichia phagocytophila* are found in abundance in polymorhonuclear cell cultures. Numerous morulae of *E. phagocytophila* were found by Jongejan *et al.* (*1*989). This was not the case when mild *Cowdria* in this study were cultivated *in vitro*. Reduced numbers of organisms in all tissues of the host may be an evolutionary compromise for co-existence between the agent and the host. Hence the usefulness of neutrophil cultures as a source of direct diagnosis and antigen production (Logan *et al., 1*987; Awa *et al.*, 1993), may be limited to the more virulent isolates unless the host is or can be made to become more susceptible. How mild strains achieve

transmission to different hosts by the vector remains unclear as such low infection rates would appear not to be self perpetuating, but perhaps endothelial and neutrophil infection is a poor guide to the infection level in an animal as other tissues could have high infection rates. It remains a matter for further investigation how mild strains are perpetuated in the field although one way could be by vertical transmission (Deem *et al., 1*996a).

The advent of the polymerase chain reaction (PCR) has made identification of bacteria in insect vectors, particularly those microbes that are difficult to culture *in vitro,* possible (Higgins, and Azad, 1995). Although establishing infection rates of *Cowdria* in ticks was not possible in this study, different isolates of *Cowdria* were detected by PCR. Not all isolates were detected with equal ease and it was suggested that there may be slight nucleotide differences in the HE region of the 16S rRNA gene. This points to a need for caution when using PCR to test different isolates of unknown *Cowdria.* Negative results may not be truly negative while positive results can be confirmed by hybridisation. Although the sequencing of the isolates were not carried out in this study, (Nicoll *et al., 1*997 in preparation), analysing the restriction fragment length of the GroE operon, showed that the Kenyan isolates were distinct from the South African and Zimbabwean isolates but that they did not all fall into one group.

It could be suggested that the mild isolates in which colonies were not observed in the brain smears at death might not be *Cowdria.* This point affects Bamba and Kathiani 32 isolates particularly. As mentioned earlier the Bamba isolate was observed in the endothelial cells of the brain biopsy before death which could indicate that these sheep might have eliminated the organisms in the brain but died of an immune related reaction. Hence the possible reason why no colonies were present at death. The Bamba isolate then fits the biological description of a *Cowdria* given all the other findings that have been made on it. On the other hand, the Kathiani 32 was the only isolate not to have colonies observed in the brain smears. While this isolate appears not to meet the biological classification of *Cowdria,* it was not investigated exhaustively perhaps, by serial biopsy, to demonstrate the total absence of colonies. Notwithstanding, it induced convincing clinical reactions in sheep, western blot patterns that were similar to its sister Kathiani 972, and most convincing it yielded a 388 bp product with the HE1(cr)/HE3(l) primers which are specific for *Cowdria.* Also a sheep recovered from

Kathiani 32 infection reacted to heterologous challenge in a similar manner to other sheep immunised with other mild isolates. Since molecular tools are now available perhaps they should be trusted to provide a more accurate definition of *Cowdria.* Although *Cowdria* and *Ehrlichia* are more closely related to one another than with other *Rickettsia* (Dame *et al.* 1992, Van Vliet *et al.* 1992), phylogenic trees constructed according to the DNA sequences of different genes have so far grouped members of genus *Cowdria* together, relating them more closely to each other than to the closest members of the genus *Ehrlichia, (E. canis, E. chaffeensis* and *E. ewingii),* (Van Vliet *et al.* 1992; Drancourt and Raoult, 1994; Nicoll *et al.* 1997). Thus considering phylogenic relatedness together with clinical and post mortem pathology, failing to observe colonies in the brain should not exclude Bamba and Kathiani 32 isolates from being regarded as *Cowdria.* Rather they should be regarded as the far limit of the spectrum which is being observed more and more as a *Cowdria* characteristic.

In immunisation, treatment on various days of fever has been shown to affect the number of casualties to the immunisation and the level of immunity (Gueye, and Vassilides, 1985). The animals were found to be at risk of either not acquiring sufficient protection if treated too early or of dying from the disease if treated too late. Lawrence *et al.,* (1995b). found that fewer deaths occurred in cattle treated on the first day of fever and variations in frequency of reactions, incubation period and severity of reaction did not affect the efficiency of immunisation, as assessed by seroconversion. In this study, immunising with the virulent isolates, such as Suswa and Baragoi but not the milder isolate, caused some deaths. This means that any virulent isolate adopted for a vaccine should be strictly monitored and treatment given early. On the other hand maintaining enzootic stability may require that intensive acaricide treatment should not be introduced (Meltzer *et al., 1*995).

Conclusions

1. Strains of *C. ruminantium* in Kenya differ in their biological properties including virulence to sheep and mice. The majority of the 10 new isolates were highly virulent to sheep and caused typical heartwater. All isolates caused seroconversion. The minority resulted in mild reactions, lower proportion of deaths and very low infection

rates in brain endothelial cells.

2. Low virulence strains of heartwater resulted in infections that were difficult to diagnose by examination of brain crush smears and by serologic tests. This could lead to under recognition of heartwater in some locations in Kenya.

3. Isolates from *A. gemma* and *A. lepidum* ticks were made. The isolates from *A. gemma* were very divergent in characteristics, one had a very high virulence and the other a low virulence. The results suggest that regions in which *A. gemma* is the principal vector will not have a decreased risk of virulent heartwater challenge.

4. High virulence isolates provided good cross protection against other high virulence stocks and also reduced the infection with low virulence strains. The results provide a significant guide to the selection of stocks for heartwater vaccination. However there may be some high virulence isolates against which protection is inadequate and perhaps these should be incorporated in a cocktail or subsequent vaccine.

5. Primary infection of low virulence did not result in protection and they appeared to increase susceptibility to virulent strains. This suggests low virulence infections initiate non protective mechanisms which lessen resistance on re-exposure. The implication is that low virulence or attenuated vaccines should be used with caution as they may be unsafe. Animals that recover from one strain of heartwater may be at risk in Kenya from another strain.

6. High virulence strains may have the optimum level of cross protection to use as vaccine but virulence may be difficult to control in susceptible sheep and possibly cattle. Characterisation of immunogenic strains that have reduced virulence is required if progress on live vaccines is required.

7. *Cowdria* infection in Kenya *Amblyomma* ticks needs to be investigated. Increase in the distribution of the tick species among susceptible livestock through changes in dipping regimes would result in increased exposure to ticks and opportunity for occurrence of heartwater. Training in diagnosis will be required for detection of infection and much research is required for identification of suitable vaccines for use in control.

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APPENDICES

APPENDIX 2.1: REAGENTS USED FOR WESTERN BLOTTING

1) **10% SDS**
10g powder
Disolve in MQ water and make to 100ml H_2O

2) **1.5M Tris HCl pH 8.8**

Tris base	18.15g

Disolve in 100 ml miliq water
Correct pH with concentrated HCl to pH 8.8
store in fridge (4 C)

3) **0.5M Tris HCl pH 6.8**

Tris base	6g

Disolve in 100ml MilliQ water.
Correct pH to 6.8 with 1N HCl.

4) **x5 Running buffer pH 8.3** (electrode buffer)

Tris base	9g
Glycine	43.2g
SDS	3g

make to 600ml with MQ water
Store at 4 C and warm to 37 C before use if precipitation occurs.

5) **Sample buffer** (SDS reducing buffer)

	x2 stock
Distilled water	8.2ml
0.5M Tris Hcl, pH6.8	5ml
Glycerol	4ml
10% (w/v) SDS	0.8g
2-b-mercaotoethanol	2ml
0.05% (w/v) bromophenol blue	spatula tipful
MilliQ water	8.2ml
Total	20ml

Store at 20 C (room temperature).

6) **Acrylamide/bis (30%T 2.67%C)**

Acrylamide	87g(29.2g/100ml)
N'N'-bis-methelene- acrylamide	2.4g (0.8g/100ml

make to 300ml with distilled water. filter and store at 4 C in the dark (30 days maximum)
(or add 2g/100ml Duolite MB6113 and stir for 15 min. then filter and store at -20 C and store in aliquots.

7) Destain (fix)

acetic acid	100ml
methanol	250ml
MilliQ water	650ml
Total	1000ml

(Remove gel background to satisfaction - usually for 1-3hrs while checking)

8) Stains

a) 0.1% Coomasie blue

Coomassie blue R-250	0.1g

Disolve in 100ml destain.

b) Silver staining

$AgNO_3$	0.19g
H_2O	100ml

As per instruction in kit. 1 hr 45min for 0.75mm gel

or

fix gel 10 min in fix solution
drain off and stain with silver stain for 10 min on rocker in darkness/semi darkness (donnot fold gel) on gentle rocker
wash x2 in tap water (mind loosing the gel!)
develop for 10 min on gentle rocker
stop rxn with 100ml stop solution
take photograph

6) Developer (100ml per two gel)

NaOH	6g	
MilliQ water	200ml.	pre-constitute and keep
Formaldehyde	1.5ml	add just before use
(NB = 4 gel)		

Stop solution

Na carbonate	15g
H_2O	2000ml

c) 0.1% amido black

0.1g amido black
Disolve in 100ml destain (for staining n-c paper).

9) Blocking buffer x10

Tris	60.5g
Sodium chloride	87.0g
EDTA	3.72g
Nonidet P40	5.0ml
Gelatin	25.0g
Thiomersal	2.0g

MilliQ water to 1000ml.
warm to disolve while stirring but do not overheat.
Change pH to 7.4 using concentrated HCl.

Dilute in milk powder (Marvel®) solution

10) Transfer buffer

(48mM Tris, 39mM glycine, 20% methanol, 1.3mM SDS (0.0375%), pH9.2)

Tris	5.82g	
Glycine	2.93g	
SDS	3.75ml	of 10% SDS in double distilled water
Methanol	200ml	

Adjust the volume to one litre with double distilled water.

11) Phosphate buffered Saline (PBS)

x10	
Sodium chloride	80.0g
Potassium chloride	2.0g
Disodium hydrogen phosphate (anhydrous)	11.5g
Potasium hydrogen phosphate	2.0g

Disolve all in 1littre of milliQ water final volume.
Autoclave at 115 C for 10 minutes. (solution pH 7.3 approx.)

12) Ammonium per sulphate (APS)

100mg in 1ml MQ water
(make fresh every day)

13) Separating gel preparation-0.375M tris pH 8.8

		12%	7.5%
Distilled water		3.5ml	4.85ml
1.5M Tris-HCl <pH 8.8		2.5ml	2.5ml
10% (w/v) SDS stock		100µl	100µl
Acrylamide/ Bis (30% stock) *		4.0ml	2.5ml
10% ammonium persulfate**		50µl	50µl (0.05%)
TEMED	+	5µl	5µl (0.05%)
TOTAL	=	10ml	10ml

* - Degas for ≥ 15 min. at room temperature
** - make fresh dailly.

2 Stacking gel preparation - 4% gel, 0.125M Tris, pH 6.8

	two gels	one gel
Distilled water	6.1ml	3.05ml
0.5M Tris-HCl pH 6.8	2.5ml	2.5ml
10% (w/v) SDS stock	100ul	50ul
Acrylamide/ Bis (30% stock)*	1.3 ml	0.65ml
10% ammonium persulfate**	50ul	25ul(0.05%)

TEMED		10ul	5ul(0.1%)
TOTAL	=	10ml	5ml

* - Degas for ≥ 15 min. at room temperature
** - make fresh dailly.

APPENDIX 2.2: REAGENTS FOR USE IN THE PCR

2 **Stock solutions**

2.1 primers

2.1.1 HE1 (M6822) provided at 144.94µM

stock solution required 2µM
= 72.5 x dilution.

For 500µl put 6.9µl into 493µl milliQ water.

2.1.2 HE3 (1) (M6825) provided at 120.14µM

stock solution required 2µM
= 60 x dilution

For 500µl put 8.3µl into 492µl MilliQ water.

2.2 Nucleotides (dNTPs)
Each of 4 dNTPs provided at 100mM

Stock solution required 2mM
= 50 x dilution.

for 500µl put 10µl of each dNTPs into 460µl of Milliq water.

2.5 TBE x 10
x10 for one litre

Tris	108 g
Boric acid	55 g
0.5 M EDTA pH8.0	40ml (to make add 93.05g in 500ml MQ)

This was used at x 1 to prepare the agarose gel and gel running buffer.

2.6 DNA loading buffer x 6

bromophenol blue	0.025 g
xylene cyanol	0.025 g
glycerol	3 ml
MilliQ water	7 ml

2.7 Ethidium bromide solution (10mg/ml)

100mg Ethidium bromide Dissolve in 10 ml MilliQ water.

3.1 Master mix used to amplify *Cowdria* DNA extracted from blood (example)

Solution	Stock	Required	for. 1 tube	For 12 tubes
MilliQ water	-	-	24.6µl	295 µl
dNTPs	2mM	0.2mM	5µl	60µl
forward primer	2µM	0.2µM	5µl	60µl
reverse primer	2µM	0.2µM	5µl	60µl
Taq buffer	x 10	x 1	5µM	60µM
Taq enzyme	5u/µl	2u/tube	0.4 µl	5 µl
Total			**45µl**	**540µl**

3.2 Master mix used to amplify *Cowdria* DNA extracted from ticks (example)

Solution	Stock	Required	for. 1 tube	For 20 tubes
MilliQ water	-	-	27.25µll	545µl
Taq buffer	x 10	x 1	5µl	100µl
MgCl2	50mM	1.5mM*	1.5µl	30µl
dNTPs	10mM	200µM	1µl	20µl
forward primer	5µM	0.5µM	5µl	100µl
reverse primer	5µM	0.5µM	5µl	100µl
Taq enzyme	5u/µl	1.25u/tube	0.25µl	5µl
Total			**45µl**	**900µl**

* based on 2.5 mM MgCl2 contributed by 1x Taq buffer

4 1.2 % agarose gel in 1 xTBE containing 0.2µg/ml ethidium bromide/

Agarose	2.4g	(1.2g for Minigel same comb)
TBE x 10	20ml	(10ml)
MilliQ water	180ml	(90ml)

5 High molecular weight DNA marker

x 6 loading buffer	2µl
MilliQ water	9µl
Mwt markers	1µl

2 **Stock solutions**

2.1 Primers-set 1 (AB 128) and (AB 129)

2.1.1 AB 128 (Cruachem Ltd, Glasgow, UK)
amino acid sequence 5´ ACTAGTAGAAATTGCACAATCTAT 3´
Provided at 11.6µM (lyophilised)
reconstitute to 10µM
= add 1.16ml (1160µl).
working stock required **5µM**
∴ take 400µl rec. + 400µl MQ

2.1.2 AB 129 (Cruachem Ltd, Glasgow, UK)
amino acid sequence 5´ TGATAACTTGGTGCGGGAAATCCTT 3´
Provided at 8µM (lyophilised)
reconstitute to 10µM
= add 0.8ml (800µl).
working stock required **5µM**
∴ take 400µl rec. + 400µl MilliQ water.

2.2 Primer set 2 (Cruachem Ltd, Glasgow, UK)

2.2.1 Avar-F1
amino acid sequence 5 TAAGGACAAGAAGACCCTAAGAAT ´ 3´
provided at 12.6µM (lyophilised)
reconstitute to 10µM
= add 1.26ml (1260µl).
working stock solution required **2µM**
∴ take 200µl rec. + 800µl MilliQ water.

2.2.2 Avar-R2 (**25086**) provided at 9.3µM (lyophilised) (Cruachem Ltd, Glasgow, UK)

amino acid sequence 5´ TAACTTCTTCATTAAATAAGAATCC 3´

reconstitute to 10µM

= add 0.93ml (930µl).

working stock solution required **2µM**

∴ take 200µl rec. + 800µl MilliQ water.

2.3 dNTPs stock solution (10mM)

Nucleotides (dATP, dCTP, dGTP, dTTP) each at 100mM

	for 200µl stock
each dNTPs	20 µl
Milliq water	120µl
Total	200µl

Dispense into 40µl volumes (x5). Store at -20 C]

2.6 TBE x 10

This is used at x 1 to prepare the agarose gel and gel running buffer.

x10 for one litre

Tris	108 g
Boric acid	55 g
0.5 M EDTA pH8.0	40ml (to make add 93.05g in 500ml MQ)

2.6 DNA loading buffer x 6

bromophenol blue	0.025 g
xylene cyanol	0.025 g
glycerol	3 ml
MilliQ water	7 ml

2.7 10mg/ml Ethidium bromide solution

100mg in 10 ml MilliQ water.

This is highly toxic, care when using.

[Used in agarose gel at 0.2 µg/ml

= 4 ul in 200ml]

2.8 Sterile MilliQ H_2O (100ml).

Ethidium bromide Staining of agarose gel

Add 4 µl of 10 mg/ml EtBr to 100ml used (x1 running) TBE buffer.

Pour over gel in a staining tray and stain for 1 hour.

TE

10 mM Tris base pH 8.0

1 mM EDTA pH 8.0

10X TBE

108 mM Tris base

55 g boric acid

40 mls 0.5 M Na_2EDTA pH 8.0

One part with nine parts miliQ water makes x1 TBE

5X DNA loading buffer

50% glycerol
100mM EDTA
1% SDS
0.1% bromophenol blue
0.1% xylene cyanolor

or

Blue Juice (Sample loading buffer) x2 stock

EDTA	0.28g
sucrose	2.5g
0.05% (w/v) bromophenol blue	spatula tipful
MilliQ water **to**	10 ml

Store at room temperature. Use 1µl sample buffer +5µl sample

Proteinase K buffer

also PK digestion buffer -

10mM Tris HCl pH 8.3
50mM Kcl
2.5mM $MgCl_2$
0.5% Tween 20
0.5% Nonidet P-40

Lysis buffer

(for ELISA antigen preparation)

0.5% NP-40
0.5% Na deoxycholate in TEN 50mM Tris pH7.4
150mM NaCl
2mM EDTA

Appendix 4.1a: Clinical Inspection Checklist

Date	Animal #	Temp	eating	resp.	Dull	noise teeth /grunting	motion	diarroea	lying down	death	t'mnt/ TLA/ other

Appendix 4.1b

Animal no.....Spp.....
Experiment.....

Date	Day	Temp.	Ticks	EDTA bld.	Serum	comments

Appendix 4.2

P.M. No. ..... Animal..... Date.....

External appearance

Mucuos membranes

Muscles

GIT

Liver

Kidney

Spleen

Lymph nodes

Lungs

Heart

Blood

Samples

Diagnosis

Signed

Appendix 4.3: To show the frequency with which morulae were seen in neutrophil cultures of different isolates during isolation and stablilate testing.

Cowdria Isolate	Sheep ID	Death/ treated	Temp. duration days	Day of T culture started	No of days cultures set up	Development of neutrophil infection during pyrexia* Days culture +ve	Proportion of febrile days +ve (≥ 1%)	Highest percent +ve (day 2 cultures only)	Highest percent +ve (all)	Comment on colonies
Suswa	41*	y	(2)	1	3	3, -, 2-4. **	2/3(1)	1	1	SM dv
	53	y	4	2	3	3, 1-3, 1.	3/3(0)	0	<1	L
	54	t*	3	2	2	0&3, 2.	2/2(0)	0	<1	ML
Baragoi	49*	y	5	2	4	-, 0-3, 1-4, 0-3.	3/4(2)	2	3	M
	40	y	4	1	4	-, 1-2, -, -.	1/4(0)	0	<1	M
	44	y	4	1	3	1-3, 1-3, -.	2/3(0)	0	<1	ML
	59	t	5	2	2	-, 3.	1/2(0)	0	<0.1	L
Kathiani	43	y	4	1	4	1-3, 1, -, 2-3.	3/4(0)	0	<1	M
972	46	t	6	1	3	2-3, 1-2, 1&3.	3/3(1)	1	1	M
	34***	n	7	1	7	-, 2-3, 1-3, 2, -, -, -.	3/7(0)	0	<0.1	M
Kiswani	33	t	6	3	1	-.	0/1(0)	0	0	
	28	y	2	2	1	0-2.	1/1(1)	25	25	SML dv
	36	y	(3)	2	2	1-2, 1-2.	2/2(1)	7	7	SM dv
	45	y	3	1	3	5, 1, 0-2.	3/3(1)	1	1	M
Galana229	30	y	3	1	3	-, -, -.	0/3(0)	0	0	
	31	(y)	(7)	5	3	-, -, 2-3.	1/3(0)	<1	<1	M
Galana233	51	(y)	3	3	2	2, 2.	2/2(0)	<1	<1	M
	61	t	4	1	3	2, 2, -.	2/3(0)	<1	<1	M
Isiolo	27*	(y)	2	2	1	-.	0/1(0)	na	0	
	38	t	6	2	2	-, -.	0/2(0)	0	0	
	39	y	8	2	6	2-4, 2-3, 2-3, -, 0-3, 3.	5/6(0)	<1	<1	M

Appendix 4.3 (cont.)

Asembo	50*	y	(2)	2	1	-.	0/1(0)	0	0	
	52*	y	(1)	1	1	0-3.	1/1(1)	5	30	SM dv
	62	t	(7)	1	3	2-3, 1-3, -.	2/3(2)	1	6	LM dv
	63	n	(8)	3	6	3, 1-3, 1-3, 1-3, 1-2, 3.	6/6(1)	<1	10	LM dv

Cowdria Isolate	Sheep ID	Death/ treated	Temp. duration days	Day of T culture started	No of days cultures set up	Development of neutrophil infection during pyrexia* Days culture +ve	Proportion of febrile days +ve (≥ 1%)	Highest percent +ve (day 2 cultures only)	Highest percent +ve (all)	Comment on colonies
Marigat	29*	(y)	6	2	5	-, -, -, -, -.	0/5(0)	0	0	
	42	y	4	2	3	-, 1-2, -.	1/2(1)	1	1	L dv
	47	n	6	2	7	1, -, 3, 1-3, 0, -, -.	4/7(0)	<1	<1	SM
	48	n	6	1	8	-, -, -, 1, -, -, -, 2-3.	2/8(0)	<1	<1	M
Bamba	34	n	(9)	1	9	-, -, -, 0-2, -, -, 4,	3/9(0)	<1	<1	M
	55	n	7	2	6	3, -.	1/6(1)	1	1	S dv
	57	t	4	1	2	2, -,-, -, -, -. 1-3, -.	1/2(1)	1	1	L
Kathiani3	32*	(y)	(7)	1	6	-, -, -, -, -, -.	0/6(0)	0	0	***
2	35	n	6	2	6	-, -, -, -, 0, -.	1/6(0)	0	<1	M
	58	n	(4)	1	4	2-3, -, 1, -.	2/4(1)	<1	1	L dv

KEY **Column 2** - * one star = sheep infected with tick stabilate. 34 This animal was seen to have an a 'morula' in the cytplasm of a monocytic cell. 32* cultures in this animal got contaminated on the third day culture that was isolated. 34*** - this was the only challenge animal whose neutrophils were cultured. Cytospins had few neutrophils, and numerous lymphocytes and macrophages. 50^{i} - The superscript denotes that this animal was an isolation animal. Others were stabilate testing animals

Column 3 - y = yes, n = no, t = treated, t* = NB no cultures made after treatment, (y) = animal euthanised during high fever but could have lived longer or possibly not died at all.

Column 4 - duration of temperature in brackets was taken from the beginning of the main peak if the pre peak was marginal (e.g. 40.3 C) or if the animal responded to non specific treatment (e.g. with Penicillin/streptomycin). The cut off level used was ≥ 40 C.

Column 7 - numbers indicate day culture had infection, given in order of days of pyrexia on which culture established ie l, -, 2-4 = infection was observed in 3rd and 5th day cultures, on the 3rd day of 3rd day culture and on the 2nd -4th day of 5th day culture. no infection in cultures established on 4th days;

Column 9 - S = small colonies, M = medium colonies, L = large colonies, dv = divided colonies, ***one cultures had bacterial contamination.

Appendix 4.4 Post mortem lesions in sheep that died of cowdriosis through out the whole study

Isolate	Sheep #	Brain colonies	Nasal & subdural congestion	Lung lesions	Hydro thorax	H/peric ardium	Heart lesions	Full g/bladder	Liver lesions	Kidney lesions	Spleen lesions	Ascites	GIT h'ges	Perineal soil	Eye lesions
Suswa	53	+	-	-	-	-	-	++	+	+	-	-	-	-	-
Suswa	41	+	-	+	-	-	-	+++	+	+	-	-	++	+	-
Suswa	15	+	-	+	-	+	+	+++	+	+	-	-	-	-	-
Suswa	92	+	-	+	+	+	+	++++	+	+	+	-	-	-	-
Suswa	136	+	-	+	+	-	++	++++	++	++	+	-	+	+	-
Suswa	134	+	-	+	+++	+	++	++++	+	++	+	-	-	+	-
Suswa	125	+	+	+++	+	-	++	++++	+	+	+	-	-	+	-
Suswa	147	+	-	+++	+	+++	++	++++	+++	+++	+	+	-	-	-
Suswa	151	+	-	+	-	-	+	+++	-	+	-	+	-	-	-
Suswa	164	+	+	+++	-	+	+	++++	+	++	+	-	-	-	-
Suswa	175	+	-	+	+	-	+++	++++	+	++	++	-	-	+	-
Suswa	122	-	-	+	+	+	+	++++	+	++	-	-	-	+	-
Suswa	156	+	+	+	+++	+	++	++++	+	+	+	+	-	-	-
Suswa	145	+	+	+	+	+	++	++++	+	+	-	-	-	+	-
Suswa	174	-	-	+++	+	-	+++	++++	+	++	+	+	-	-	-
Suswa	165	+	-	+	+	+	+	+++	+	+	+	-	-	+	-
Suswa	116	++	-	+	+	-	+	+++	+	+	+	+	-	-	-
Suswa	124	+	-	++	+	-	+	++++	++	++	+	++	-	+	+
{Suswa}	35	+	-	+	-	+	-	++	+	+	-	-	-	+	-
{Suswa}	63	++	-	+	++	+	-	-	+	+	-	+	+	-	-
{Suswa}	5	+	+	+	-	+	+	++++	+	+	-	+	-	+	-
{Suswa}	11	+	+	+	-	-	+	++++	+	+	+	-	-	+	-
{Suswa}	12	+	-	+	+	+	-	++++	+	+	+	-	-	+	-
{Suswa}	73	+	+	+	+	-	+	++++	+	+	+	-	-	+	-
{Suswa}	2	+	+	+	+	+	+	++++	+	+	+	-	-	+	-
{Suswa}	70	+	-	+	+	-	+	++++	+	+	-	+++	-	+	-
{Suswa}	107	+	-	+	-	+	++	+++	+	+	-	-	-	-	-
{Suswa}	108	+	-	+	+	+	++	++++	+	+	+	++	-	-	-

Appendix 4.4 (cont.)

Isolate	Sheep #	Brain colonies	Nasal & subdural congestion	Lung lesions	Hydro thorax	H/peric ardium	Heart lesions	Full g/bladder	Liver lesions	Kidney lesions	Spleen lesions	Ascites	GIT h'ges	Perineal soil	Eye lesions
{Suswa}	115	++	+	+	+	+	++	+++	+	+	+	-	+	-	-
Baragoi	49	++	+	+	-	-	-	++	-	++	+	-	-	-	+
Baragoi	40	++	++	+	+	+	+	++++	++	++	+	+	+	-	-
Baragoi	44	++	-	-	+	+	-	+++	-	-	-	-	-	-	-
Baragoi	66	++	-	+	+	+	+	+++	-	+	-	-	+/-	-	-
Baragoi	100	+	-	+	+	+	+	++++	+	+	-	-	-	-	-
Baragoi	133	+	-	+++	+	+	++	++++	++	++	+	+	+	-	-
Baragoi	166	++	-	++	++	-	+	+++	+	+	+	-	+/-	-	+
{Baragoi}	20	+	-	+	+	+	+	++++	+	+	+	+	-	-	-
{Baragoi}	113	++	+	+	++	+++	++	++++	++	+	+	+	-	-	-
{Baragoi}	67	+	+	+	+	+	+	++++	+	+	+	+	+/-	-	-
{Baragoi}	69	+	-	+	+	+	+	+++	+	+	-	-	-	-	-
{Baragoi}	85	++	-	+	+	+	-	-	+	+	+	-	-	-	-
{Baragoi}	7	+	-	+	+	+	++	++++	++	++	+	+	-	-	-
{Baragoi}	71	+	-	+	+	-	++	++++	+	+	+	++	-	-	-
{Baragoi}	88	++	-	++	+	+	++	++++	++	+	+	+	-	-	-
{Baragoi}	95	++	-	+	+	+	++	++++	+	++	+	-	-	-	-
{Baragoi}	112	+	-	+	+	+	+	+++	+	+	+	-	-	-	-
Kat 972	972	+++	-	+++	++	++	-	-	+	+	+	-	-	-	-
Kat 972	43	+++	-	-	+	+	-	+++	+	+	-	+	+	-	-
Kat 972	98	++	-	+	+	+	+	++++	+	+	-	+	-	-	-
Kat 972	103	++	-	+	-	+	-	+++	+	+	+	-	-	-	-
{Kat 972}	58	+	-	++	+	-	+	++++	+++	++	-	-	-	-	-
{Kat 972}	14	+	-	+	-	-	+	++	+	+	+	+	-	-	-
{Kat 972}	16	+	-	++	+	+	++	++++	++	+	+	-	-	-	-
{Kat 972}	65	+++	-	+++	+	+++	+	++++	++	++	+	-	-	-	-
{Kat 972}	74	++	-	+	+	+	+	++++	+	++	+	-	-	-	-
{Kat 972}	93	++	+	+	+	+	+	++++	+	+	+	-	-	-	-
{Kat 972}	9	+	-	+	++	+	+	++++	+	+	-	-	-	-	-

Appendix 4.4 (cont.)

Isolate	Sheep #	Brain colonies	Nasal & subdural congestion	Lung lesions	Hydro thorax	H/peric ardium	Heart lesions	Full g/bladder	Liver lesions	Kidney lesions	Spleen lesions	Ascites	GIT h'ges	Perineal soil	Eye lesions
{Kat 972}	19	+	-	+	+	+	+	+++	+	+	-	+	-	-	-
{Kat 972}	68	++	+	++	+	+	+	+++	-	+	+	+	-	-	-
{Kat 972}	90	++	-	+++	-	+++	+	++++	+	+	+	+	-	-	-
Kiswani	28	+	-	+	+	+	-	++++	+++	++	-	-	+	-	-
Kiswani	36	+	-	+	+	+	-	+++	+	+	-	-	+	-	-
Kiswani**	45	++	-	+	-	+	-	-	-	-	+	-	+	-	-
{Kiswani}	177	+++	-	+	-	+	+	++++	++	++	-	-	-	+	+
Ga 229	229	++	-	+	-	-	-	++	-	-	+	-	+++	-	-
Ga 229	30	..	-	-	+	+	+	-	+	+	+	-	-	-	-
Ga 229	31	++	++	+	-	+	+	++++	+	-	-	-	+++	-	+++
Ga 233	51	++	-	+	+	-	-	++++	+	+	-	-	-	-	
Asembo	50	++	-	++	+++	++	+	++++	-	+/-	-	+	-	-	-
A%*sembo	52	+++	-	-	-	-	-	++	-	+	-	-	-	-	-
Asembo	84	+	+	++	+++	+	+	++++	++	++	+	+	-	-	-
Asembo**	72	+	-	++	+	+	+	++	+	+	-	-	-	-	-
{Asembo}	87	+	+	++	+++	+	+	++++	+	+	+	-	-	-	-
Marigat	29	-	-	+	+	+	-	++++	+	-	+	-	-	-	-
Marigat	42	+	-	+	+	-	-	+++	-	++	-	-	+	-	-
Marigat	22	+	-	+	+	+	-	++++	+++	+++	-	+	-	-	-
Isiolo	27	+++	-	-	+	+	-	-	+	-	-	-	-	-	-
Isiolo	39	++	-	+++	+++	-	-	++++	-	++	-	-	-	-	-
Bamba	129	-	++	+	++++	-	+++	++++	+++	+++	+++	-	-	-	+++
Bamba	243	-	-	++	+	+	+	-	+	+++	-	+	+	-	+++
Kat 32	32*	-	-	-	+	+	-	-	-	+	-	-	-	-	-

KEY

{} - immune animal challenged with this isolate; ** mouse organ homogenates used to infect animalAppendix 4.4 Infection Rate in Endothelial cells of brain capilaries and Neutrophil Cultures from cross immunity and tick infectivity animals

Appendix 4.5 (cont.)

Isolate	Sheep #	Brain colony count in 400 endothelial cells (%)	Highest count of morulae on day 2 culture in 400 neutrophil cells (%)	Highest count of morulae in 400 neutrophil cells (if other) (%)	Isolate	Sheep #	Brain colony count in 400 endothelial cells(%)	Highest count of morulae on day 2 culture in 400 neutrophil cells(%)	Highest count of morulae in 400 neutrophil cells (if other) (%)
Suswa	115	37(9.25)	nd		Baragoi	117	na	0	
Suswa	116	24(6)	0		Baragoi	118	na	0	
Suswa	119	30(7.5)	0	2(0.5)	Baragoi	123	na	0	
Suswa	124	4(1)	nd		Baragoi	126	na	0	8(2)
Suswa	125	2(0.5)	17(4.25)		Baragoi	127	34(8.5)	1(0.25)	
Suswa	134	5(1.25)	0		Baragoi	128	na	+ve	
Suswa	135	na	0		Baragoi	131	38(9.5)	nd	
Suswa	141	na	0		Baragoi	138	na	32(8)	
Suswa	143	na	1(0.25)		Baragoi	139	na	34(8.5)	
Suswa	149	na	1(0.25)		Baragoi	142	na	2(0.5)	
Suswa	151	+ve	13(3.25)	14(3.5)	Baragoi	146	na	2(0.5)	
Suswa	159	na	0		Baragoi	157	na	0	+ve
Suswa	160	na	0		Baragoi	158	na	nd	
Suswa	163	na	0		Baragoi	161	21(5.25)	0	5(1.25)
Suswa	169	7(1.75)	nd		Baragoi	162	na	7(1.75)	
Baragoi	170	na	2(0.5)		Baragoi	166	21(5.25)	0	2(0.5)
Baragoi	172	na	0		Baragoi	167	na	0	
Baragoi	173	na	0		Baragoi	168	na	4(1)	5(1.5)

KEY

This table includes all the sheep that died and those that did not die but were sampled for neutrophil cultures. Animals have been differentiated by putting a star for those that were challenge animals and indicating if the neutrophil cultures were done during the first infection or challenge infection.

Appendix 4.6a Titration of pre-infection and post infection sera by the celisa test obtained from heartwater infected sheep used to carry out western blots

Dilution	Control sera neg/pos	Sheep 35 Kath. 32 4399/4772	Sheep 58 Kath. 32 5015/5584	Sheep 47 Marigat 4458/4773	Sheep 48 Marigat 4296/4472	Sheep 54 Suswa (t) 4813/5416
neat	3.5/**93.1***	9.2/**94.2**	10.3/**93.9**	12.5/**96.9**	45.8/**92.1**	9.4/**94.1**
1/2	0/**89.3**	3.3/**87.3**	2.8/**89.3**	4.7/**94.2**	36.2/**85.5**	4.8/**90.2**
1/4	0/**82.0**	0/**79.6**	1.3/**82.9**	7.2/**92.4**	27,3/**77.5**	2.6/**82.3**
1/8	0/**72.5**	0/**66.9**	2.4/**72.4**	0.6/**89.2**	20.7/**67.4**	7.8/**73.7**
1/16	0/**62.3**	0/**53.9**	7.7/**62.1**	0.6/**84.9**	17.6/**58.3**	1.5/**57.3**
1/32	0/46.0	0/40.4	5.6/**54.3**	2.3/**77.3**	10.5/**51.5**	0/38.9
1/64	0/31.9	0/29.1	5.6/42.7	0/**67.7**	15.5/40.8	0.3/24.9
1/128	0/4.2	0.3/13.3	1.6/31.8	0/49.8	1.5/36.0	0/7.1

Appendix 4.6b: Titration of pre-infection and post infection sera by the celisa test obtained from heartwater infected sheep used to carry out western blots

Dilution	Control sera neg-pos	Sheep 61 Gal 233 (t) 5986-6131	Sheep 31/239 Gal. 229 (t) 2908- *	Sheep 38 Isiolo (t) 4098-4098	Sheep 33 Kiswani (t) 2959-4097	Sheep 46 Kath 972 (t) 4786-5205
neat	0-**96**	0/**90.6**	11.6/**83.5**	54.7/**95.6**	3.1/**76.7**	0/**84.5**
1/2	0/**91.1**	0/**76.2**	0.6/**69.9**	31.5/**90.1**	0/**60.0**	0/**65.0**
1/4	0/**81.8**	0/**56.5**	0/**53.1**	1.5/**85.6**	0/44.0	0/48.2
1/8	0/**67.2**	0/38.0	0/38.4	16.2/**79.7**	0/31.3	0/26.7
1/16	0/**56.1**	0/23.8	0/26.	5.8/**70.4**	0/18.4	0/11.4
1/32	0/43.4	0/16.5	0/13.1	0/**55.9**	0/4.9	0/0.2
1/64	0/27.0	0/13.2	0/5.0	0/40.1	0/1.5	0/0
1/128	0/12.2	0/0	0/0	0/27.1	0/0	0/0

Appendix 4.6c: Titration of pre-infection and post infection sera by the celisa test obtained from heartwater infected sheep used to carry out western blots

Dilution	Control sera. neg-pos	Sheep 62 Asembo (t) 5206-5935	Sheep 63 Asembo 5207-5936	Sheep 55 Bamba 4832-5582	Sheep 34 Bamba 4455-4771	Sheep 59 Baragoi (t) 5775-6129
neat	6.8/**93.7**	18.3/**83.2**	9.3/**90.8**	9.4/**94.8**	14.1/**93.2**	10.8/**96.2**
1/2	8.1/**87.4**	6.4/**69.3**	0/**84.7**	3.4/**89.7**	2.0/**87.3**	2.6/**92.7**
1/4	0/**79.8**	2.8/**55.0**	0/**57.6**	3.8/**82.8**	0/**81.6**	2.3/**86.1**
1/8	0/**67.2**	0/45.5	0/**66.5**	2.4/**73.5**	0/**73.5**	0/**77.9**
1/16	0/**54.5**	0/35.9	0/**55.6**	5.2/**61.9**	0/**68.6**	0/**67.8**
1/32	0/44.1	0/28.7	0/42.1	7.5/47.3	0/**64.8**	0/**56.9**
1/64	0/27.2	0/19.1	0/30.5	3.5/31.8	0/**56.9**	0/46.6
1/128	0/15.3	7.0/23	1.5/31.2	6.2/21.5	2.4/**54.1**	0/35.6

KEY (for Tables 4.9a, b, & c)

NB - each column has the result of pre and post infection sera separated by a / in that order, e.g. 3.5/93.1* - pre-infection serum - 3.5% inhibition/ post-infection serum - 93.1% inhibition in the cELISA test

neg - Negative control serum; pos - Positive control serum

Statistics

Appendix 4.7: The statistical significance of mortality between groups of sheep infected with different isolates: p values

Isolate										
Suswa										
Baragoi	-									
Kat. 972	-	-								
Kiswani	-	-	-							
Ga 229	-	-	-	-						
Ga 233	-	-	-	-	-					
Isiolo	-	-	-	-	-	-				
Asembo	-	-	-	-	-	-	-			
Marigat	-	-	-	-	-	-	-	-		
Bamba	<0.05*	<0.01	<0.05	-	-	-	-	<0.05	-	
Kat 32	<0.05	<0.05	<0.05	-	-	-	-	-	-	-
Isolate	Suswa	Baragoi	Kat. 972	Kiswani	Ga 229	Ga 233	Isiolo	Asembo	Marigat	Bamba

KEY

- not significant

<0.05* - p <0.05 = there is a 95% probalility that the difference in mortality between the Suswa and Bamba infected animals is not by chance but because of a real difference in their virulence.

Appendix 4.8 Temperature reaction of isolation animals

	Isolate	Suswa	Suswa	Baragoi	Kat 972	Ga 229	Ga 233	Isiolo	Asembo	Asembo	Marigat	Marigat	Bamba	Kat 32
	Day pi	sh 965	sh 41	sh 49	sh 972	sh 229	sh 233	sh 27	sh 50	sh 52	sh 245	sh 29	sh 243	sh 32
source	sheep no.	nn	guts	guts	guts	nn	nn	guts	guts	guts	guts	guts	aa	guts
	0	39.6	38.6	39	38.8	38.9	38.9	39	nd	nd	39.1	38.8	38.9	39
	1	39.4	40.3	40.8	39.1	38.6	39.2	39.4	38.6	39.4	39.1	38.6	39.3	39.7
	2	39.4	39.9	39.6	38.7	39	39.3	39.2	39.2	39.2	38.8	39.7	39	38.7
	3	39.6	39.9	38.8	38.7	38.7	39.4	39.5	39.5	38.8	40.5	38.7	39.3	39.1
	4	39.2	39.9	39.3	38.9	38.8	39.3	39.6	39.7	39.1	40	38.5	39	39
	5	39	39.9	38.9	39.5	39	39	39.6	40	39.5	39.7	39.5	38.5	40.6
	6	38.9	39.7	39	38.5	38.8	38.9	39.5	39.7	39.6	39.7	39.3	39.3	39.9
	7	39.4	40.6	40.3	39	38.5	39.1	39	40.1	39.5	40.1	38.5	39	40
	8	38.8	40.6	40.6	39.6	39.1	39.3	39.4	39.9	40	39.2	39.2	39.1	39.8
	9	39.6	41.2	41.5	39	39	39	39.1	39.5	39	39	39.6	38.8	39.3
	10	40	39.5	41.7	38.7	38.4	39.3	39	40	38.5	38.5	39	39	39.6
	11	40	39.3	40.6	40.7	38.9	39.5	38.8	39.5	39.2	39.6	39.6	38.8	40.1
	12	39.9	40.6	c*	41.3	39.5	38.7	40.6	40.5	39.7	39.5	39.5	39.2	40
	13	39	40.7		40.3	39.4	38.8	41.5	40.6	41.1	39.4	39.1	40.2	40.1
	14	39.3	39.2		c*	38.4	39.3	c**	f*	c*	39.5	40.2	40.3	40.5
	15	40.1	c*			38.5	39.9				39.5	41	40.4	41.2
	16	39.6				37.9	41				40.5	41.6	38.8	41.4
	17	39.7				37.9	41.1				41.8	41.6	39	41.4
	18	41.3				38.6	f*				41.7	40.8	39.8	41
	19	42				38.5					c*	40.9	39	c*
	20	c*				40.7						c*	39	
	21					40.6							39.1	
	22					38.5							f*	
	23					c*								

Appendix 5.1: Summary of experiment one: outcome of immunisation of naive sheep with the Asembo and Bamba isolates, their boost and heterologous challenge and their serological status on the cELISA test after each proceedure,

Phase	Immunisation							Boost			Heterologous challenge								Serology			
Imm isolate	Sh. no	Inc.	Pf	D to Pf	D of F	D. rec.	OTC	Boost	Pf	D of F	D of chal.	challenger	Incub	Pf	D to Pf	D of F	death	recovery	Pre-inf	Post-inf	Post-boost	Post-het
Asembo	74	11	41	12	4	15	12	32	40.2	3	72	Kath 972	10	41.9	13	4	13	-	25.1	77.9	83.5	*
Asembo	93	11	41.8	13	7	18	13	32	-	-	72	Kath972	12	41.9	13	3	14	-	56.1	87.7	89.2	*
Asembo	65	9	41.7	13	5	16	13	32	-	-	72	Kath972	10	41.8	10	3	12	-	22.1	82.9	86.2	*
Asembo	16	12	41.8	13	5	17	14	32	40.1	1	72	Kath972	12	42	13	5	16	-	34.3	83.3	92.7	*
Asembo	14	11	42	13	5	16	13	32	40.1	2	72	Kath972	10	42	13	5	14	-	36.4	79.6	91.2	*
Asembo	94	11	42	13	4	15	13	32	40.8	4	72	Bamba	23	40.2	24	2	-	25	27.5	84.0	53.3	90.0
Asembo	64	12	42	13	4	16	13	32	-	-	72	Bamba	15	40.7	16	5	-	20	27.6	79.6	89.2	89.8
Asembo	6	11	41.9	13	6	17	13	32	41	9	72	Bamba	na	na	na	na	-	-	17.0	84.3	83.3	87.9
Asembo	96	11	42	13	5	16	13	32	-	-	72	Bamba	na	na	na	na	-	-	45.1	88.3	91.6	93.0
Asembo	10	13	41.9	14	4	17	14	32	41	2	72	Bamba	22	40.1	22	3	-	25	18.8	76.3	nd	90.3
Asembo	67	12	41.8	13	4	16	13	32	40.3	1	72	Baragoi	10	41.8	12	5	14	-	40.8	84.1	84.2	*
Asembo	113	10	42	12	5	15	12	32	40.2	4	72	Baragoi	9	41	10	3	11	-	29.2	82.2	87.9	*
Asembo	82	13	40.9	13	2	15	14	32	40	1	72	Baragoi	died	-	-	-	-	-	48.7	90.5	na	-
Asembo	69	11	42	14	5	16	14	32	40	2	72	Baragoi	11	41	14	4	14	-	48.7	83.8	78.8	*
Asembo	85	11	42	13	5	16	13	32	40.5	4	72	Baragoi	10	41.6	13	4	13	-	30.6	89.6	61.4	*
Asembo	73	13	42	13	4	17	14	32	40.5	5	72	Suswa	9	40.7	9	1	9	-	32.9	88.5	79.4	*
Asembo	24	10	42	12	5	15	12	32	40.8	4	72	Suswa	9	42	13	8	-	17	50.7	83.9	85.4	91.4
Asembo	5	13	41.8	14	4	17	14	32	41	6	72	Suswa	9	41.7	9	1	9	-	30.0	76.9	81.7	*
Asembo	11	11	42	13	5	16	13	32	40.1	4	72	Suswa	8	40.5	9	2	9	-	24.9	83.2	87.8	*
Asembo	12	11	42	12	4	15	12	32	-	-	72	Suswa	6	41.6	10	5	10	-	20.0	83.5	86.3	*
Bamba	19	16	40.8	20	4	21	20	43	-	-	72	Kath 972	11	40.6	13	3	13	-	27.7	87.7	92.2	*
Bamba	68	17	41.4	20	3	21	20	43	-	-	72	Kath 972	11	41.6	13	3	13	-	31.0	81.5	87.8	*
Bamba	90	20	40.5	20	5	25	21	43	-	-	72	Kath 972	13	41.8	13	3	15	-	33.5	69.5	93.8	*
Bamba	80	16	40.5	20	5	21	20	43	-	-	72	Kath 972	13	41.6	13	6	-	19	27.5	83.0	82.6	89.4
Bamba	9	15	42	16	3	18	16	43	-	1	72	Kath 972	10	42.7	13	5	14	-	25.5	85.4	90.7	*
Bamba	17	15	41.6	17	4	20	18	43	-	-	72	Asembo	13	40.8	14	3	-	16	29.9	79.2	79.2	88.1
Bamba	87	16	40.7	16	4	21	21	43	40.5	3	72	Asembo	11	41.7	13	4	14	-	31.2	22.1	23.2	*
Bamba	79	14	41.6	17	4	19	18	43	-	-	72	Asembo	13	40.1	23	3	-	24	34.7	81.1	85.1	89.0
Bamba	89	17	40.8	20	5	22	20	43	-	-	72	Asembo	13	41.6	16	4	-	17	31.9	84.4	88.5	92.2
Bamba	99	24	41.1	26	3	27	26	43	-	-	72	Asembo	-	-	-	-	-	-	25.8	73.9	84.1	83.0
Bamba	88	14	41.8	17	7	21	17	43	40	1	72	Baragoi	10	41.2	11	3	12	-	40.9	87.9	78.3	*
Bamba	7	-	-	-	0	-	none	43	-	-	72	Baragoi	9	41.8	12	5	13	-	17.0	31.9	29.6	*
Bamba	71	15	42	18	4	20	18	43	-	-	72	Baragoi	10	41.7	12	4	13	-	39.0	81.6	87.8	*
Bamba	112	15	41.6	18	5	20	18	43	40	2	72	Baragoi	10	41.8	12	6	15	-	20.6	87.8	79.6	*
Bamba	95	14	42	16	7	21	16	43	-	-	72	Baragoi	10	41.8	11	3	12	-	25.6	84.2	82.9	*
Bamba	70	14	41	18	5	19	18	43	-	-	72	Suswa	none	-	-	0	9	-	27.1	90.0	86.5	*
Bamba	107	14	41.7	16	5	19	16	43	-	-	72	Suswa	8	41.5	10	4	11	-	47.0	91.7	88.4	*
Bamba	115	15	41.9	16	5	20	16	43	40.5	1	72	Suswa	10	41.6	12	3	12	-	24.4	81.4	87.7	*
Bamba	2	15	40.2	16	5	20	21	43	-	1	72	Suswa	8	41.6	9	2	9	-	41.1	74.9	66.9	*
Bamba	108	14	40.9	16	4	18	16	43	-	-	72	Suswa	10	42	13	6	15	-	26.6	82.1	90.2	*

KEY: imm. isolate - immunising isolate; Sh # - sheep number; Inc – incubation; dash (-) = no reaction ; Post-het - post haterologous titre
OTC - day of treatment with oxytetracycline; D to Pf - day to peak fever; D of f - days of fever ; Pf - peak fever; Post-boost - post boost infection titre
Boost - day inoculated with homologous stabilate; D rec - day to recovery ; D of Chal. - day of challenge; Pre-inf. - preinfection titre
challenger - challenging isolate; death - day to death ; Post-inf. - post infection titre

Appendix 5.2 Summary of experiment two: outcome of immunisation of naive sheep with the Baragoi and Suswa isolates, their boost and heterologous challenge and their serological status on the cELISA test after each proceedure

Phase	Immunisation							Boost			Heterologous challenge								Serology			
Immuniser	Sh #	Inc	Pf	D to Pf	D of f	D to rec	OTC	boost	Pf	D of f	D of Chal.	challenger	Incu	Pf	D to Pf	D of f	death	D to rec	Pre-inf.	Post-inf.	Post-boost	Post-het
Baragoi	117	10	41.3	12	5	15	11	41	-	-	62	Asembo	13	40.8	13	2	-	17	17.9	78.6	8 .1	86.8
Baragoi	118	9	41.3	10	5	14	10	41	-	-	62	Asembo	-	-	-	-	-	-	29.3	79.3	90.9	84.5
Baragoi	154	9	41.5	10	3	12	10	41	41.3	3	62	Asembo	-	-	-	-	-	-	18.7	81.5	89.4	87.9
Baragoi	167	9	41.2	9	5	15	12	41	-	-	62	Asembo	-	-	-	-	-	-	18.4	71.2	: 5	89.8
Baragoi	172	11	41.7	12	4	15	12	41	-	-	62	Asembo	-	-	-	-	-	-	18.1	71.0	85.8	84.1
Baragoi	139	8	41.3	9	5	13	10	41	-	-	62	Bamba	-	-	-	-	-	-	17.3	77.4	86.5	80.2
Baragoi	142	10	41.1	10	4	14	11	41	-	-	62	Bamba	-	-	-	-	-	-	16.9	73.3	85.2	87.6
Baragoi	162	9	41.4	11	7	16	11	41	40	1	62	Bamba	-	-	-	-	-	-	13.9	78.3	87.7	86.7
Baragoi	168	10	41.4	13	5	15	11	41	40.2	1	62	Bamba	-	-	-	-	-	-	42.3	78.8	90.1	86.0
Baragoi	173	11	41.8	12	5	16	12	41	40.2	2	62	Bamba	-	-	-	-	-	-	55.9	82.1	88.4	82.8
Baragoi	86	10	41.3	10	5	15	10	29	40.1	-	49	Suswa	22	40.1	22	1	-	23	18.5	63.6	86.2	89.3
Baragoi	126	9	41.6	10	4	13	10	41	40	-	62	Suswa	-	-	-	-	-	-	2.3	72.4	93.5	87.6
Baragoi	128	9	41.1	9	3	11	10	41	-	-	62	Suswa	-	-	-	-	-	-	15.7	71.8	91.9	91.6
Baragoi	146	8	41.7	11	5	13	10	41	-	-	62	Suswa	-	-	-	-	-	-	7.0	81.9	87.2	79.2
Baragoi	157	9	41.3	10	4	13	10	41	40.6	-	62	Suswa	-	-	-	-	-	-	18.0	75.8	86.9	70.9
Baragoi	123	8	41.1	10	5	13	10	41	40.2	-	62	Kath 972	21	40.4	21	1	-	22	35.5	82.6	92.2	84.2
Baragoi	138	11	41.3	11	4	15	11	41	-	-	62	Kath 972	-	-	-	-	-	-	41.9	80.5	87.9	74.4
Baragoi	148	10	41.5	11	4	14	11	41	-	-	62	Kath 972	-	-	-	-	-	-	39.7	69.4	85.3	53.9
Baragoi	158	8	40.8	10	4	12	10	41	-	-	62	Kath 972	-	-	-	-	-	-	31.1	79.2	89.4	49.6
Baragoi	170	10	41.5	11	4	14	11	41	-	-	62	Kath 972	-	-	-	-	-	-	52.4	84.4	83.1	73.1
Suswa	3	8	41.5	8	3	12	8	31	-	-	51	Asembo	12	41.8	13	10	-	23	34.8	72.8	87.0	80.4
Suswa	105	8	41.1	10	5	13	8	31	40.5	2	51	Asembo	13	40.9	15	5	-	18	14.1	72.7	85.0	77.8
Suswa	144	8	40.6	14	5	15	10	31	40	1	51	Asembo	13	41.8	16	6	-	19	19.1	81.6	86.2	44.6
Suswa	150	8	41.9	10	4	12	9	41	-	-	62	Asembo	14	41.5	16	7	-	21	43.2	80.7	82.1	87.7
Suswa	153	-	-	-	-	-	8	31	-	-	51	Asembo	12	41.7	13	7	-	19	55.1	69.4	80.8	90.6
Suswa	8	7	40.9	7	1	9	7	31	-	-	51	Bamba	16	40.7	16	1	-	17	20.5	75.7	85.0	89.1
Suswa	13	7	41	7	2	9	7	31	-	-	51	Bamba	-	-	-	-	-	-	23.7	76.3	90.4	86.6
Suswa	141	13	40.5	13	1	14	13	30	-	-	50	Bamba	-	-	-	-	-	-	63.2	79.3	68.3	72.6
Suswa	143	8	41.8	9	6	13	9	31	-	-	51	Bamba	-	-		-	-	-	18.4	83.3	89.6	86.3
Suswa	159	8	41.6	9	4	12	9	41	-	-	62	Bamba	-	-	-	-	-	-	35.5	85.3	90.6	90.8
Suswa	101	8	41	8	3	11	8	31	40	1	51	Baragoi	15	41.8	16	5	-	20	16.6	75.9	88.5	91.3
Suswa	111	8	40.6	8	2	10	8	31	-	-	51	Baragoi	14	41.7	16	5	-	19	19 2	68.3	80.1	87.2
Suswa	149	8	41.4	9	4	12	9	41	-	-	62	Baragoi	12	41.8	14	7	-	19	14.0	75.2	82.1	nd
Suswa	160	13	41.7	13	4	17	13	30	40.1	1	50	Baragoi	14	41.5	17	7	-	21	60.4	87.5	81.5	89.8
Suswa	163	8	41.6	10	4	12	9	41	40	1	62	Baragoi	14	40.6	21	6	-	23	56.6	81.5	82.1	87.4
Suswa	104	8	41.1	10	5	13	8	31	40.6	2	51	Kath 972	14	41.3	15	2	-	16	9.2	52.4	56.9	49.6
Suswa	121	9	41.8	10	4	13	10	41	40.2	1	62	Kath 972	20	40	20	2	-	22	13.6	76.7	90.0	87.0
Suswa	132	14	40.8	15	3	17	13	30	-	-	50	Kath 972	-	-	-	-	-	-	62.9	88.4	86.2	71.3
Suswa	135	8	41.6	9	4	12	9	41	-	-	62	Kath 972	14	41.5	17	6	-	22	21.9	72.2	78.0	60.1
Suswa	137	-	-	-	-	-	8	31	-	-	51	Kath 972	15	40.2	15	1	-	16	65.1	78.1	88.1	85.1

KEY: imm. isolate - immunising isolate; Sh # - sheep number; Inc – incubation; dash (-) = no reaction
OTC - day of treatment with oxytetracycline; D to Pf - day to peak fever; D of f - days of fever ; Pf - peak fever
Boost - day inoculated with homologous stabilate; D rec - day to recovery ; D of Chal. - day of challenge
challenger - challenging isolate; death - day to death ; Post-inf. - post infection titre
Pre-inf. - preinfection titre; Post-het - post haterologous titre; Post-boost - post boost infection titre

Appendix 5.3 The reaction indices of sheep immunised and challenged with different combinations of *Cowdria* isolates

First experiment						Second experiment					
sheep #	Immuniser	challenger	RI (imt)	RI (hob)	RI (hech)	sheep #	immuniser	challenger	RI (imt)	RI (hob)	RI (hech)
17	Bamba	Asembo	6.14	1.37	2.54	117	Baragoi	Asembo	8.6	0	2.32
79	Bamba	Asembo	6.36	0	2.14	118	Baragoi	Asembo	8.5	0	-0.74
87	Bamba	Asembo	2.18	1.96	48.36	154	Baragoi	Asembo	6.71	4.46	0
89	Bamba	Asembo	4.15	0.86	4.96	167	Baragoi	Asembo	4.42	0	0
99	Bamba	Asembo	3.74	0.88	0.78	172	Baragoi	Asembo	3.3	0	0
81	naive control	Asembo	0	50.94		140	naive control	Asembo	0	0	50.4
91	naive control	Asembo	2.18	50.73		171	naive control	Asembo	0	0	48.56
20	naive control	Asembo	1.16	0	21.75	144	Suswa	Asembo	5.29	0	11.7
84	naive control	Asembo	1.96	0.72	45	150	Suswa	Asembo	7.38	0	12.38
6	Asembo	Bamba	7.38	6.38	1.9	153	Suswa	Asembo	0	0	11.23
10	Asembo	Bamba	7.34	2.02	1.91	3	Suswa	Asembo	6.34	0	19.3
64	Asembo	Bamba	6.76	0	6.22	105	Suswa	Asembo	9.55	2.14	7.1
94	Asembo	Bamba	5.06	6.55	1.34	139	Baragoi	Bamba	8.3	0	0
96	Asembo	Bamba	8.34	0	0.7	142	Baragoi	Bamba	7.27	0	0
83	naive control	Bamba	1.29	17.25	0	162	Baragoi	Bamba	12.34	4.21	0
102	naive control	Bamba	3.55	0.52	0.67	168	Baragoi	Bamba	6.3	7.63	0
23	naive control	Bamba	0.83	0	18.03	173	Baragoi	Bamba	9.85	5.52	0
97	naive control	Bamba	0	0.94	13.24	120	naive control	Bamba	0	0	2.3
20	Asembo	Baragoi	21.75	na	50.24	129	naive control	Bamba	0	0	44.14
67	Asembo	Baragoi	7.04	1.08	46.22	141	Suswa	Bamba	1.4	0	0
69	Asembo	Baragoi	8.9	1.37	45.9	143	Suswa	Bamba	9.52	0	0
82	Asembo	Baragoi	4.1	0.8	na	159	Suswa	Bamba	7.84	0	0
85	Asembo	Baragoi	7.35	2.31	37.46	8	Suswa	Bamba	1.5	0	1.61
113	Asembo	Baragoi	8.25	2.44	47.57	13	Suswa	Bamba	3.54	0.9	0
7	Bamba	Baragoi	0	0	47.6	127	naive control	Baragoi	0	45.64	na
71	Bamba	Baragoi	8.76	0.77	49.16	166	naive control	Baragoi	0	50.7	na
88	Bamba	Baragoi	8.63	1.36	44.16	161	naive control	Baragoi	0	0	51.25
95	Bamba	Baragoi	8.71	0	49.06	131	naive control	Baragoi	0	0	50.21
112	Bamba	Baragoi	6.96	0	41.99	149	Suswa	Baragoi	7.2	0	11.39
66	naive control	Baragoi	0	0	50.06	160	Suswa	Baragoi	6.7	0	10.97
100	naive control	Baragoi	3.76	0	49.42	163	Suswa	Baragoi	7.1	0.6	4.01
14	Asembo	Kath 972	8.25	1.27	46.5	101	Suswa	Baragoi	5.58	0	11.44
16	Asembo	Kath 972	10.35	1.08	46.85	111	Suswa	Baragoi	3.26	0	9.58
65	Asembo	Kath 972	8.6	0	51.08	123	Baragoi	Kath 972	8.95	1.35*	1.45
74	Asembo	Kath 972	3.72	2.19	48.62	138	Baragoi	Kath 972	9.7	0	0
93	Asembo	Kath 972	9.89	0	51.53	148	Baragoi	Kath 972	7.18	0.98*	0
9	Bamba	Kath 972	6.03	1.98	50.2	158	Baragoi	Kath 972	5.04	0	0

Appendix 5.3 (cont.)

First experiment						Second experiment					
sheep #	Immuniser	challenger	RI (imt)	RI (hob)	RI (hech)	sheep #	immuniser	challenger	RI (imt)	RI (hob)	RI (hech)
19	Bamba	Kath 972	4.6	0	48.17	170	Baragoi	Kath 972	6.56	0	0
68	Bamba	Kath 972	4.81	1.68	50.04	152	naive control	Kath 972	0	0	49.56
80	Bamba	Kath 972	4.9	1.51	12.9	155	naive control	Kath 972	0	0	48.42
90	Bamba	Kath 972	3.5	0	49.76	121	Suswa	Kath 972	7.46	0.92	1.56
18	naive control	Kath 972	0	1.08	50.3	132	Suswa	Kath 972	4.63	0	0
103	naive control	Kath 972	0	0	47.35	135	Suswa	Kath 972	7.46	0	5.48
5	Asembo	Suswa	5.56	3.51	53.28	137	Suswa	Kath 972	0	0	1.06
11	Asembo	Suswa	8.95	4.62	52.46	104	Suswa	Kath 972	9.6	2.03	2.37
12	Asembo	Suswa	7.2	0	45.9	126	Baragoi	Suswa	7.36	8.4	0
24	Asembo	Suswa	6.5	4.5	15.09	128	Baragoi	Suswa	4.42	0	0
73	Asembo	Suswa	6.94	2.88	52.17	146	Baragoi	Suswa	8.7	0	0
2	Bamba	Suswa	2.85	1.01	52.81	157	Baragoi	Suswa	5.14	2.33	0
70	Bamba	Suswa	6.4	0	54	86	Baragoi	Suswa	5.3	0.72	0.62
107	Bamba	Suswa	8.8	0	48.83	116	naive control	Suswa	0	50.42	na
108	Bamba	Suswa	3.78	0	46.3	124	naive control	Suswa	0	51.48	na
115	Bamba	Suswa	8.65	3.19	5.41	119	naive control	Suswa	0	0	45.82
15	naive control	Suswa	0	0.52	52.02	169	naive control	Suswa	0	0	52.73
92	naive control	Suswa	0	0	51.73						

Key: RI (imt) - reaction index during immunisation (temperature reaction); na - not applicable
RI (hob) - reaction index during boosting (temperature reaction)
RI (hech) - reaction index during heterologous challenge (total outcome)
* - due to temperature after inoculationbefore incubation

Appendix 5.4 The reaction of control sheep infected with different *Cowdria* isolates

	Column	2	3	4	5	6	7	8	9	10	11
								Day to		Serology	
	Isolate	Sh. #	Period	Incu	D of f	P f	p f	Dth	Rec	Pre-inf	Post-inf
Exp. 1	Asembo	81	ho	12	3	41.6	14	15	-	34.2	*
	Asembo	91	ho	12	3	42	14	14	-	27.9	*
	Asembo	84	he	9	7	42	13	15	-	20.2	*
	Asembo	20	he	11	15	41.8	13	-	26	22.3	82.6
	Bamba	102	ho	na	na	na	na	-	na	14.1	11.5
	Bamba	83	ho	17	12	41.9	21	-	28	34.7	85.4
	Bamba	23	he	16	9	41.6	19	-	25	28.1	89.2
	Bamba	97	he	19	7	41.5	22	-	26	28.1	81.8
	Baragoi	100	he	9	5	42	13	13	-	26.6	*
	Baragoi	66	he	8	6	41.9	12	13	-	16.3	*
	Kath 972	103	he	9	5	41.6	10	13	-	37.0	*
	Kath 972	98	he	10	5	42	12	14	-	10.1	*
	Suswa	15	he	9	2	41.6	10	10	-	17.3	*
	Suswa	92	he	8	3	42	10	10	-	24.3	*
Exp. 2	Baragoi	127	ho	9	7	41.7	13	15	-	31.2	*
	Baragoi	166	ho	10	5	41.7	12	14	-	53.7	*
	Baragoi	131	he	10	4	41.3	12	12	-	16.9	*
	Baragoi	161	he	9	5	41.9	12	13	-	51.6	*
	Suswa	116	ho	9	3	41.4	10	12	-	68.6	*
	Suswa	124	ho	9	2	41.1	10	10	-	33.5	*
	Suswa	119	he	7	6	41.9	10	12	-	51.6	*
	Suswa	169	he	10	1	41.2	10	10	-	20.7	*
	Bamba	120	he	17	3	40.3	17	-	20	24.4	80.9
	Bamba	129	he	14	8	41.6	19	21	-	20.7	*
	Asembo	140	he	12	3	41.6	12	14	-	53.2	*
	Asembo	171	he	10	6	41.9	12	15	-	56.8	*
	Kath 972	152	he	10	4	41.9	13	13	-	36.1	*
	Kath 972	155	he	10	4	41.7	13	13	-	48.9	*

KEY

Sh. # - sheep number; Dth – death; * - dead; ho - homologous boost
Incu – incubation; Rec – recovery; , he - heterologous challenge
) of f - days of temperature Pre-inf – preinfection; -/na - not applicable
P f - peak fever; Post-inf - post infection
Period - phase of the cross-immunity trial during which this animal was infected,
Serology* - % inhibition on the cELISA test (mean of two plates, positive cut off 70%)

Appendix 5.5: Serology

Two Sample T-Test

Isolate	Comparison	T	P	DF	95% C.I.	Note
Baragoi	Pre-inf. vs Post-inf.	14.58	0.0001	24	58.3 - 43.9	ES
	Pre-inf. vs Post-boost	18.55	0.0001	20	69.4 - 55.36	ES
	Pre-inf. vs Post-het.	13.23	0.0001	35	63.4 - 46.5	ES
	Post-inf. vs Post-boost	8.09	0.0001	30	14.1 - 8.41	ES
	Post-inf. vs Post-het.	1.37	0.18	27	9.6 - 1.9	NS
	Post-boost vs Post-het.	2.81	0.01	21	1.93 - 12.9	VS
Suswa	Pre-inf. vs Post-inf.	8.97	0.0001	24	53.3 -33.3	ES
	Pre-inf. vs Post-boost	10.25	0.0001	25	59.5 -39.6	ES
	Pre-inf. vs Post-het.	8.4	0.0001	34	57.6 -35.2	ES
	Post-inf. vs Post-boost	2.46	0.019	37	11.4 -1.1	S
	Post-inf. vs Post-het.	0.84	0.41	28	10.6 - 4.5	NS
	Post-boost vs Post-het.	0.86	0.4	28	4.4 - 10.8	NS
Asembo	Pre-inf. vs Post-inf.	18.38	0.0001	23	55.8 - 44.53	ES
	Pre-inf. vs Post-boost	14.09	0.0001	35	56.8 - 42.5	ES
	Pre-inf. vs Post-het.	21.39	0.0001	21	62.6 - 51.53	ES
	Post-inf. vs Post-boost	0.22	0.83	21	4.79 - 5.9	S
	Post-inf. vs Post-het.	6.03	0.0001	20	9.28 - 4.51	NS
	Post-boost vs Post-het.	2.98	0.0078	19	12.7 - 2.21	NS
Bamba	Pre-inf. vs Post-inf.	10.72	0.0001	25	55.6 - 37.7	ES
	Pre-inf. vs Post-boost	10.69	0.0001	24	58.3 -39.4	ES
	Pre-inf. vs Post-het.	26.03	0.0001	14	62.7- 53.2	ES
	Post-inf. vs Post-boost	0.37	0.71	37	14.1 - 9.7	NS
	Post-inf. vs Post-het.	2.62	0.016	22	20.2 - 2.4	S
	Post-boost vs Post-het.	2.01	0.057	22	18.5 - 0.3	NS

KEY

Pre-inf. - preinfection
Post-inf. -post infection
Post-boost - post boost infection
Post-het. -post heterologous challenge

ES - extremely significant
VS - very significant
S - significant
NS - not significant

Appendix 5.6 Temperature record of sheep in the first experiment

Day	Date	sh. 2	sh. 5	sh. 6	sh. 7	sh. 9	sh. 10	sh. 11	sh. 12	sh. 14	sh. 15	sh. 16	sh. 17
0	23-Oct	39.4	39.5	39.7	39.3	39.4	39.1	39.4	39	39	39.6	39.1	39.3
1	24-Oct	39.9	39.4	39.6	39.8	39.2	39.7	39.6	39.5	39.7	39.6	38.9	39.5
2	25-Oct	39.9	39.6	39.9	39.7	39.6	40	40.1	39.7	40.1	40.3	39.6	39.8
3	26-Oct	39.5	39.5	40.1	39	39.1	39.8	39.9	39.3	40	40	39.7	39.2
4	27-Oct	39.2	38.8	39.9	38.9	38.9	39.5	39.7	39.1	39.9	39.8	39.3	39.5
5	28-Oct	39.8	40.6	39.5	39.4	39.6	39.7	39.3	39.4	39	39.7	38.9	39.4
6	29-Oct	39	39.7	39.5	39.6	39.3	39.5	39.5	39.5	39.4	39.8	39	39.6
7	30-Oct	39.4	39	39.5	39.5	38.9	39.5	39.4	39.6	39.8	39.8	39.2	39.3
8	31-Oct	39.5	39.1	39.6	39.4	39.2	39.3	39.2	39.6	39	39.7	39.3	39.5
9	1-Nov	39.9	39.4	39.6	39.3	39.1	39.9	39	39.4	38.9	39.8	40.3	39.4
10	2-Nov	39.2	39.3	39.5	39.1	38.8	39.8	39.2	39.9	39.5	39.4	39.8	39.2
11	3-Nov	39.9	39.4	**40.1**	39.4	39.6	39.4	**40.4**	**41.2**	**40.1**	40.1	39.1	39.8
12	4-Nov	39.9	39.7	41.2	38.8	39.4	39.6	41.5	42	41.3	40	40.3	39.7
13	5-Nov	39.8	**40.4**	41.9	38.5	39.8	41.5	42	41.5	42	39.8	41.8	39.4
14	6-Nov	39.7	41.8	40.8	39.2	39.1	41.9	41.4	40.5	41.3	39.6	41.9	39.6
15	7-Nov	39.5	40.4	41.4	39.4	39.7	41.3	41.1	39.3	41.2	39.5	42	40.6
16	8-Nov	**40.1**	40.8	40	39.5	**41.4**	41.2	39.7	38.9	39	39.6	41.2	39.8
17	9-Nov	40.2	39.9	39.2	39.8	42	39.6	39.5	39.5	39.4	39.8	39.8	**41.6**
18	10-Nov	40.2	39.3	38.8	39.5	40.5	39.1	39.4	39	39.6	39.8	39.4	41.6
19	11-Nov	40.2	39.7	39.3	39.2	39.8	39.2	39.3	39	39.7	39.8	39.2	40.3
20	12-Nov	40.2	39.5	39.2	39.7	39.5	39.3	40	39.4	39.6	39.6	38.9	39.6
21	13-Nov	39.6	39.9	39.4	39.5	39.4	39.3	39.4	39.1	39.5	39.9	38.9	39.5
22	14-Nov	39	39.6	39.3	39	39.4	39	39.6	39	39.4	39.8	39.6	39.4
23	15-Nov	39.9	39.3	39.3	39.3	39.1	39.1	39.3	38.9	39.6	39.6	38.9	39.3
24	16-Nov	38.6	39	39.3	38.4	39	38.9	39	39.1	39.5	39.7	38.3	39.3
25	17-Nov	39.9	38.9	39.4	38.9	39.2	39	39.6	38.9	39.7	39.4	38.9	39.5
26	18-Nov	39.8	39	39.5	39.8	39.4	39.9	39.6	39.4	38.7	39.5	38.5	39.2
27	19-Nov	39.5	39.7	40	39	39.6	39.1	39.2	39.7	39.1	39.5	38.9	39.7
28	20-Nov	39.9	39.7	40.2	39	39.1	39	39.6	39.9	39.2	39.6	39.3	39.6
29	21-Nov	40.3	39.9	39.5	39.2	39	39.1	39.4	39.4	40	39.5	39	39.5
30	22-Nov	39.9	39.8	39.6	39.6	39.8	38.8	39.5	39.3	39.7	39.5	39	39.3
31	23-Nov	39.8	39.8	39.9	40	40	38.5	39.6	39.3	39.1	39.4	39.2	39.5
32	*24-Nov*	*40*	*40*	*40.2*	*39.3*	*40.3*	*39.4*	*40*	*39.6*	*39.3*	*39.6*	*39.5*	*40*
33	25-Nov	39.9	39.9	40.8	39.8	39.8	39	39.8	39.8	39.9	40	39	39.6
34	26-Nov	39.7	40	41	39.6	39.7	39.3	39.8	39.2	39.6	39.9	39.1	39.3
35	27-Nov	39.8	39.2	40.9	38.7	38.9	39.6	39.5	39	39	39	38.5	39.7
36	28-Nov	39.6	39.2	41	39	39.2	39.1	39.4	39.1	39.7	39.4	38.3	39.4
37	29-Nov	39.2	39.6	41	39	39.2	39	39.3	39.7	39.2	39	38.6	39
38	30-Nov	39.3	39.8	40.1	38.8	39.2	39.1	40	39.1	39.3	39.9	38.7	40.2
39	1-Dec	38.7	40	39.3	38.9	38.9	38.5	39	38.9	39	39.1	38.4	39
40	2-Dec	38.9	41	39.3	39.4	38.9	39	38.8	39.3	39	39.3	39	39.5
41	3-Dec	39.6	40.5	40.6	39.2	39.2	39.1	39.6	39.6	39.6	39.7	39.1	39.7
42	4-Dec	39.9	39.6	39.7	39	38.9	39.2	39.2	39.1	39.6	39.6	39	39.2
43	*5-Dec*	*39.5*	*40*	39.7	*39.6*	38.9	*39.5*	*39.6*	*38.9*	*39.4*	*39.4*	39	*39.7*
44	6-Dec	39.8	39.9	39.9	39.3	38.9	39.4	39.3	39.1	39.4	39.3	39.2	39.2
45	7-Dec	39.2	39.6	40	39.3	39.1	40.3	39.2	39.5	39.3	39.7	39.7	39.1
46	8-Dec	39.3	39.4	39	39.9	38.9	41	39.5	39.6	38.7	39.7	39.4	39.7
47	9-Dec	40.1	39	39.9	38.5	39.4	39.9	39.7	39.5	39.6	39.9	40.1	39.6
48	10-Dec	39.7	39.3	39.3	39.3	38.8	39.7	39.7	39.4	40	39.5	39.5	39.4
49	11-Dec	39.5	39	39.6	39.2	39	39.3	40	39.3	39.3	39.5	39.5	39.3
50	12-Dec	39.6	39.6	39.7	39.1	38.4	39.1	40.1	39.2	39.3	39.3	39.4	39.3
51	13-Dec	39.6	39.6	39.6	39.1	38.9	39.4	39.5	39.4	39.8	39.2	39	38.6
52	14-Dec	39.9	39.9	39	39.3	39.4	39.2	39.5	39.2	40.1	39.4	38.2	39.5
53	15-Dec	39.7	39.8	39.4	39	39.4	39	39.1	38.8	39.5	39.4	38.4	39.3
54	16-Dec	39	38.4	39	38.5	39.4	38.5	39.5	38.9	38.9	39.3	38.9	39.2
55	17-Dec	39.4	39.7	39	39.6	38.9	39.5	39.8	39	39.8	39.2	39	39.3
56	18-Dec	39.5	39.5	39.3	39.6	38.9	39	39.6	38.9	39.5	39.1	39.1	39.4
57	19-Dec	39.4	39.5	39.5	39.5	38.5	38.7	39	38.7	39.6	39.2	38.6	39.1
58	20-Dec	39.5	39.3	38.8	39.4	38.9	39.3	39.2	39.2	39.7	39.5	38.6	39.2
59	21-Dec	39.4	39.5	38.9	38.8	40	38.9	39.5	39.5	39.4	39.6	38.4	39.2
60	22-Dec	38.9	39.3	39.4	38.9	39	39.3	39.5	39.3	39.5	39.3	38.9	39.2
61	23-Dec	39.5	39.1	39.8	38.8	39	39.3	39.2	39.3	39	39.9	38.6	39.4
62	24-Dec	39.6	39.5	39.5	39.1	39.6	39.6	39.3	39	39.5	39.6	38.7	39.7
63	25-Dec	39.4	39.7	39.5	39.1	39.4	39	39.3	39.4	38.9	39.6	39.1	39.7
64	26-Dec	39.7	39.2	39.5	39.1	39.5	39.7	39.9	39.4	39.6	39.8	39.5	39.7
65	27-Dec	39.3	39.4	39.4	38.7	39.2	39	39.9	39.1	39.3	39.1	38.7	39.4
66	28-Dec	39.5	39.6	39.6	38.9	39	38.9	39.8	39	38.8	39.2	38.8	39.3
67	29-Dec	39.6	39.3	39.6	38.9	39	39.5	40.4	39	39.3	39.3	39	39.5
68	30-Dec	39.6	39.2	39.7	38.6	38.9	39.1	39.9	38.8	39.4	39.5	39.4	39.3
69	31-Dec	39.7	39.7	39.9	39.2	39	39.5	40.4	39	39.5	39.7	39.3	39.7
70	1-Jan	39.5	39	39.7	39.3	39.4	39.5	40.4	39.4	39.6	39.5	39	39.7
71	2-Jan	39.6	38.8	39	39.1	39.4	38.8	39.8	39.3	39.6	39.2	39.6	39.7
72	*3-Jan*	*39.2*	*38.9*	*39.2*	*38.5*	*39.3*	*39.1*	*39.8*	*39.3*	*39*	*39.5*	*39*	*39.4*
73	4-Jan	38.9	39	39.7	39.2	39.5	39.3	39.6	39	38.9	39.5	39.6	39.4
75	5-Jan	40.2	39.4	39.3	39.8	39.2	39.9	40	39.7	39	39.2	39.2	40
76	6-Jan	39.9	39.2	39.6	39	39	39.3	40.4	39.5	39.6	39.4	39.9	39.9
77	7-Jan	39.6	39.5	39.6	39.1	38.9	40.1	40.6	39.7	39.4	39.4	38.9	40.6
78	8-Jan	39.9	39.2	39.9	39.1	39.5	39.3	40.1	39.6	39.5	39	39.5	39.8
79	9-Jan	39.9	39.8	39	39.2	39.7	39.1	40	40.3	38.6	39.6	39.6	40
80	10-Jan	39.7	39.5	38.9	39.1	39.8	39	39.6	41.3	38.9	39.4	39.5	39.7
81	11-Jan	40.2	39.7	39.4	39.1	39.6	39.5	40	41	39	39.6	39.7	40
82	12-Jan	41.6	41.7	39.3	40	39.2	39	41.5	40.2	39.5	41.2	39.3	39.9
83	13-Jan	*	*	39.8	40.8	40.2	39.6	*	41.6	40.2	41.6	39.6	40.2
84	14-Jan			39.6	41.2	41.5	39.4	*		40.4	*	39.8	39.7
85	15-Jan			38.7	41.8	41.7	39.2			40.7		40.3	39.9
86	16-Jan			40.1	40.8	42.7	39.8			42		42	40.7
87	17-Jan			39.5	*	42	39.5			40		41.9	40.8
88	18-Jan			39.3	*		39.1		*			40.3	40
89	19-Jan			39.4			39.2					40.9	39.9
90	20-Jan			39.4			39.2				*		39.2
91	21-Jan			39.7			39.3						39.6
92	22-Jan			39.5			39.3						39.2
93	23-Jan			39.9			39.5						39.1
94	24-Jan			39.7			39.5						38.6
95	25-Jan			40			40.1						39.6
96	26-Jan			39.8			40						40.1
97	27-Jan			40			40.1						39.6
98	28-Jan			39.9			39.8						39.8
99	29-Jan			39.3			39.1						38.8
100	30-Jan			39.1			39.8						39.6
101	31-Jan			39.4			39.7						39
102	1-Feb			38.3			38.4						38.9
103	2-Feb			38.4			38.6						39.1

sh. 18	sh. 19	sh. 20	sh. 23	sh. 24	sh 64	sh. 65	sh 66	sh 67	sh 68	sh 69	sh 70	sh 71	sh. 73
39.6	39.2	39.3	38.8	39.3	39.5	39	39.8	38.6	39.6	38.5	39	39.4	39.5
39.5	39.7	39.4	39.8	39.5	39.6	39.3	39.5	39	39.5	39.2	39.3	38.9	40
39.8	39.9	40	39.8	39.8	39.4	39.7	39.5	39.8	39.3	39.4	39.4	39.6	39.5
39.4	39	39	39.4	39.6	39.6	39.5	40	39.6	39.6	39	39.4	39.2	39.7
39.3	39.2	39.3	39.7	38.8	39.1	38.6	39.2	39	39	38.9	38.5	39.4	39.4
39.3	38.9	39.5	39.5	39.6	39.2	39.5	39.6	39.2	38.8	39.2	39.4	39.2	39.5
39.3	39.7	39	39.2	39.4	39.6	39.3	39.7	39.3	39.8	39	39	38.6	39.6
39.5	38.5	39.6	39.9	39.4	39.7	39.3	39.2	39.1	39.7	38.8	39.1	39	39.1
39.6	38.8	39	39	39.3	39.8	39	39.1	38.9	39.4	39.1	39	38.8	39.4
39.6	39.4	40	39.1	39.4	39.9	40.2	39.6	39.1	39.1	38.9	38.7	39.1	39.8
39.6	39.2	39.5	39	**40.4**	39.5	40.4	39.5	39.4	38.9	39.7	38.8	38.9	40.1
39.6	39.7	39.8	39.9	40.4	39.8	39.8	39.6	39.2	38.9	**40.1**	39.3	39.4	39.9
39.5	39.5	39.6	38.5	42	**41.5**	**41.3**	39.2	**41.4**	38.9	40.3	39.5	39.6	39.7
39.3	39.8	38.9	39	40.2	42	41.7	39.4	41.8	39	40.9	39.7	38	**42**
39.9	40	39.8	39.5	40.6	40.7	39.9	39.4	40.8	39	42	**40.2**	39.1	42
38.6	38.9	39.9	39.2	39.8	40.8	41.4	39.8	40	39	41.2	40	41.6	41.1
39.4	40.3	39.6	39.4	39.3	39.8	39.8	39.3	39	39.7	39.9	40.3	39.7	40.4
40.4	40.4	39.9	40.2	39.4	39.4	39.6	39.4	39.2	40.2	38.6	40.7	**40.7**	39.8
39.6	39.9	40	39.6	38.9	39.4	39.3	39.3	38.8	39.7	39.2	41	42	39.6
39.6	**40.5**	39.4	39.2	39.3	39	39.3	39.6	39.1	**40.9**	39.3	39.9	40.7	39.4
40.1	40.8	40.2	39.6	39.4	39.5	39.6	39.4	39.4	41.4	38.8	39.4	39.7	39.3
39.8	39.6	38.8	39.6	39.1	38.9	39.2	39.5	39.4	39.5	39.3	38.9	38.9	39.6
39	38.7	39	39.7	38.9	39.2	39.5	39	39.5	39.5	39	39	38.9	39.4
39.9	39.1	38.8	39	39.6	38.9	39.3	39.6	39.5	38.8	39.3	39.7	39.4	39.5
39	39.2	38.6	38.9	39.2	38.9	38.8	39.2	39.1	39	39.5	38.9	39.5	39.3
39.2	39	39	39.1	39.3	38.9	39	39.3	39.7	39.7	39.2	39	39.4	39.6
39.3	39.5	39.4	39.7	39.3	39.2	38.8	39.8	39.2	39.3	39.1	38.8	39.1	39.3
39.7	39.6	39.2	39.6	39.4	38.5	39.1	39.7	40.1	39.3	39.3	39.3	39	39.3
39	39	39.4	39.8	39.5	39.6	39	39.4	39.3	39.6	39.5	39	38.9	39.8
39.1	38.8	39.3	39.7	39.6	39	39	39.9	39	38.7	39.5	38.9	39.1	39.2
38.9	38.8	39.4	39.6	39.8	38.9	39	39.9	38.9	39.4	39.7	39	39	40.1
39.6	39.3	39.3	40	39.6	39.4	39.3	39.6	39.6	40.2	39.3	39.3	39.7	40.6
40.1	*39.3*	*39.1*	*39.9*	*39.7*	*39.5*	*39.5*	*39.7*	*40.3*	*39.5*	*39.3*	*39.5*	*40*	*40.2*
39.6	39	39.4	39.3	39.7	39.5	39.3	39.7	39.5	39.9	39.5	39.5	39.6	40
39.4	39	39.3	39	39.8	39.6	39.2	39.7	39	39.7	39.6	39.4	39	39.8
39.4	39.9	39.3	39.3	39	39.1	39.3	39.4	39.3	39	39.8	38.8	39.6	39.6
39.2	39.8	38.9	39.4	39.2	38.7	38.7	39.4	38.8	38.5	39.6	39.1	39.4	40.5
39.4	38.6	38.7	39.1	39.1	39.3	39.1	39	39.2	39.7	40	39.3	39.2	40
39.3	39.1	38.9	39.4	39.2	39	39.2	38.8	38.9	40.1	39	39.3	38.8	40.3
39	38.3	38.9	38.9	39	39	38.8	38.8	39.6	38.8	38.9	38.9	39.2	39.6
38.8	38.9	39.1	39.6	38.7	39.6	39.4	39.4	39.6	39.9	39.4	39.2	39	39
39.3	39.1	39.2	39.2	39.6	39.4	39.5	39.5	39.5	40.3	39.2	39.4	39.2	39.8
39.6	39.1	39.1	39.5	39.4	39.6	39.5	39.3	38.7	39.8	40	39	39.5	39.9
39	*39.2*	*39*	*38.8*	*39.5*	*39.5*	*39*	*39.2*	*39.7*	*39.4*	*39.8*	39.5	*39.1*	*39.6*
39.4	*39.2*	39.4	39.7	39.6	39.4	*39*	39.4	39.5	39	39.6	39	39.5	39.6
39.5	38.8	39.4	39.2	39.5	39.5	*39*	39.8	38.9	39.8	39.2	39.2	39.4	39.3
38.5	39.4	39.7	39	39.7	39.5	38.9	39.9	39	39.2	39.1	38.9	39	39.7
39.9	38.4	39.2	39.2	39.7	39.1	39.1	38.6	39	39.4	38.5	39	39	39.7
38.5	38.8	39	39.5	40.4	39.2	39.1	39.1	39	39.4	39	39.3	39.3	39.5
39	39	39.4	39.7	40.8	39	39.2	39	39.9	39	39	38.8	39.6	39.7
39.5	39	38.9	39.2	40.3	39.3	39.3	39.2	38.8	39.5	39.1	39.6	39.4	39.3
39.5	38.3	39	39.2	40.6	39.3	39.2	38.7	39.5	39.3	39.2	38.3	38.6	39.6
38.6	39.2	39	39.7	39.7	39.4	39	39.2	38.8	38.8	39.3	39	38.9	39.6
39.1	38.8	39.8	39	39	38.8	39	39	39	38.9	38.9	38.9	39.2	39.2
38.6	39	38.9	39.2	38.9	39.2	38	38.5	38.5	38.4	39.2	38.5	38.9	39.3
39.2	39.3	39.5	39.5	39	39.2	39	39.4	39.4	39.2	38.9	39	39.5	39.5
39.6	39.2	39.4	38.8	39.3	39.4	38.8	39.3	39.1	39.3	39.2	39	38.7	39.6
38.5	38.4	38.6	39.4	39.2	38.6	38.8	38.7	39.1	38.9	38.6	38.7	38.8	39
39.5	38.4	39.2	39.2	39.4	39.6	38.9	39.2	39.6	39.6	38.8	38.8	39.4	39.3
38.9	38.7	39.1	39	38.9	39.2	38.9	38.9	38.9	39	39.3	39	38.4	39.1
39.2	38.5	39	39.5	39.3	38.8	38.7	38.9	39.1	38.8	39.2	38.5	39.2	39.5
39.2	39	38.9	39.2	39.1	39.1	38.8	39.1	39.4	39.5	39.3	39	39.2	39.1
39.4	39	39.2	39.4	39.2	39	39.1	39.1	39.5	39.6	39.1	39.5	39	40
39.5	39.1	39.6	39.2	39.9	39.4	38.9	39.2	38.8	39	39	39.2	39	39.7
39.6	39.2	39.3	39.9	39.9	39.5	39.2	39.6	39.4	39.7	39.2	39.2	39.4	39.5
39	39	39.1	39.1	39.6	38.8	39	39.3	39.2	39.1	39	38.8	39	39.2
38.8	39	39.1	39.1	39.2	39.4	39	39.2	39	38.9	38.9	38.6	39.2	39.3
39.5	38.9	39	39	38.9	39.1	39	39	39	39	39.1	38.9	39.1	39.3
39.1	38.8	38.8	39.6	39.3	39.2	38.9	38.8	39	38.7	38.8	39.1	39.3	38.9
39.4	39.6	39.3	39.5	38.9	39.5	39.3	39.3	39.6	38.8	39.2	39.1	39.3	38.7
39	39	39.3	39.7	39.6	39.5	39.5	39.4	39.5	39.6	39.1	39.2	39.6	39.6
38.9	39.6	39.6	39.5	39.6	39.8	39.3	39.6	39.3	39.4	39	38.9	39	39.7
38.9	***39.2***	***38.9***	***39.1***	***39.6***	***39.5***	***39***	***38.6***	***38.7***	***39***	***38.7***	***39***	***39***	***39.2***
39	39.4	39.6	39	39.4	39.1	39.5	39	39	39	39.4	38.7	39.9	39.2
38.9	39.2	39.5	39	39.5	39.6	39.3	38.6	39	39	39	39	38.7	39.5
40.7	39.4	39	38.8	39.9	39.5	39.7	39.1	39	39.4	39.1	39.5	39.1	39.6
41.6	39.8	39.4	39.1	39.8	40	39.4	39.5	39.6	40	39	39.9	39.3	39.9
41.2	39.6	39.2	39	39.9	39.8	39.5	39.8	39.8	39.6	38.6	39	39.1	39.6
41.2	38.9	38.8	39.1	39.2	39	39	39	39.7	39.7	39.3	39.4	39.8	39.6
41.8	39	38.6	38.9	39.2	39.1	39.1	39	39.3	39.5	39.2	39.6	39.6	39.4
41.9	38.7	39.2	40.2	39.8	39.2	39.4	40.7	39	39.5	39.7	39.4	39.6	39.4
41.7	39	39.5	39.6	41	39	39.2	41.5	39.1	39.5	40.4	39.8	39.1	40.7
39.5	39.1	39.5	39.9	41.8	39.7	41.7	41.6	40.7	39	39.3 *		40.4 *	
40	40	40.5	39.3	41.5	39.6	41.4	41.5	40.9	41.5	40.5		41.5	
40.3	40.2	41.6	39.9	41.6	39.5	41	41.9	41.8	40.2	40.2		41.5	
40	40.6	41.8	39	42	39.2 *		41.7	41.7	41.6	40.6		41.2	
39.9 *		41.5	39.5	41	39.8	*		40.1 *		41	*		
39.5		41.6	39.7	41.8	40			40.7	*				
39.4		41.7	40.1	40.7	40.7		*						
39.2		40.2	41	39.8	40.7								
38.6		40.1	40.6	39.8	40.2								
39		40.5	41.6	39.5	40.6								
38.9		40	41.5	38.7	39.9								
39		40.2	41.2	39.3	39.9								
39.5		40.3	41.4	39.5	40.1								
39.4		40	41.5	40	40								
40		40	40	39.7	39.6								
39.5		40.2	39.4	39.5	39.5								
39.7		39.2	38.9	39.6	39.5								
39.5		39.6	39.4	39.5	39.4								
39.4		39.7	39.5	39.6	39.2								
38.8		39.1	39.2	38.7	38.4								
38.7		39.3	38.9	38.6	38.3								

sh. '74	sh. 79	sh. 80	sh. 81	sh. 82	sh. 83	sh. 84	sh. 85	sh. 87	sh. 88	sh. 89	sh. 90	sh. 91	sh. 92
39.7	39.5	39.1	39.3	39	39.6	39.8	39.2	39.3	39.1	38.4	39	38.9	39.1
39.4	39.5	39.6	39.5	39	39.7	39.8	39.9	40	39.6	39.6	39.5	39	39.2
39.6	40	39.5	39.2	39.6	39.6	39.3	40.1	40.4	40	39.8	39.4	40	39.7
40.1	39.4	39.3	39.6	40	40	40.2	39.6	40	39.8	39.3	39.3	39.8	40.2
39.7	39	39.1	39.1	39.1	39.3	39.3	39.3	39.1	39.7	40	39.3	39.6	39.7
39.2	39.3	39.7	39.1	39.3	39.4	38.9	39.7	39.7	39.2	39.8	39.4	39.3	39.2
39.6	38.9	39.2	39.6	39	39.4	38.9	39.4	40	39.2	39	39.4	39.2	39.1
39.8	39.2	39.4	39.2	39.1	39.4	39.1	39.3	39.6	39.2	39	39	39.7	38.9
39.5	39	39.3	39	38.9	39.5	39	39.6	39.4	39	38.9	38.7	39.5	39
39.4	39.3	39.4	39.3	39	39	39.1	39.5	39.7	39.7	39.7	39.5	39.8	39
39.9	39.3	38.7	39.3	39	39.4	39	39.1	39.8	39.4	39.7	38.4	39.7	39.2
40.7	40.1	39.6	39.5	38.9	39.5	39.8	**40.2**	39.8	39.8	39.5	39.6	39.8	39.8
41	39.5	38.4	39.1	39.5	39.2	39.7	40.6	39.6	39.2	39.6	39.3	39.6	39.7
40.1	39.8	39.7	39.4	**40.9**	38.4	39.9	42	39.9	39.8	39.8	39.8	40	39.7
40.4	40.6	39.3	39.3	40.7	39.8	39.2	41.5	39.9	40.9	39.9	39.6	39.8	39.5
39.6	40.8	39.9	39.3	39.8	39.3	40.7	40.8	39.8	40.4	39.6	39.5	40.5	39.5
39.7	39.1	40.1	39.5	40.1	39.7	38.8	39.5	**40.7**	41	39.2	39.5	40	39.2
40.4	41.6	40.3	39.8	39.6	40.7	40	39.7	40.3	41.8	40.2	39.4	39.7	39.4
39.4	41	40.4	39.7	39	39.4	39.5	39.4	39.4	40.4	40.1	39.8	40	39
40	39.7	40.3	39.5	39.3	39.9	39.4	39.2	40.1	40.4	40.5	39.3	40.2	39.2
40.2	39.5	40.5	39.7	39.6	39.7	39.4	39.3	40.2	40.3	40.8	40.5	39.9	39.3
39.6	39.9	39.8	39.2	39.3	39.4	39.3	39.8	39.1	39.9	40.2	40.3	39.9	39.6
39	39.2	39.6	39.1	39.3	39.2	39.3	39.7	39.1	39.7	38.9	40	39.4	38.8
39.8	39.6	39.5	39.4	38.9	39.4	39.8	39.4	39.6	39.4	39.8	40.1	39.8	38.7
39.5	38.5	39.1	39.3	38.9	39.3	39.4	39.5	39.4	39.5	39.5	39.7	39.3	38.9
39.6	39.3	39.4	39	39	39.4	39.8	39.7	39.9	39.9	39.7	39.7	39.4	38.5
39.4	38.6	38.6	39.3	39.2	39.5	39	39.1	39.5	39.9	39	39	39.5	39.5
40.1	39.1	39.6	39.4	39.1	39.6	40.2	39.8	39.5	39.9	39.5	39.4	40	39.2
39.8	39.1	39.2	39.4	39.3	39.8	39.3	39	39.7	39.8	39.2	39	39.6	40
39.7	39	39.5	39.5	39.3	39.7	38.7	39.4	39.9	39.9	39.9	39.3	39.8	39.7
39.3	39	40	39	39.3	40	39.3	39.5	39.3	39.9	39.8	39.7	39.9	39.1
39.4	39.3	39.7	38.8	39.5	39.5	39.4	39.4	39.7	39.6	39.9	40.5	39.6	39.7
39.6	*39.7*	*39.7*	*39.5*	*40*	*39.6*	*39.7*	*39.7*	*39.6*	*39.9*	*39.8*	*39.9*	*39.8*	*39.7*
40	38.9	39.9	39.2	39.5	39.7	39.8	39.4	39.3	39.9	39.7	39.7	39.5	39.5
40	39	39.6	39.1	39.5	39.6	39	39.4	39.2	39.7	39.5	39	39.8	39.5
39.2	38.8	39.4	39	39	38.9	39	39.6	39.9	38.9	39.4	39.7	39.8	38.7
39.9	38.8	39.2	40	38.7	39.5	39	39.8	39.6	39.7	39.3	39.5	40.4	39
39	39	39.1	39.7	38.8	39.6	39	40.5	39.4	40	40.3	39.3	39.5	38.7
39.7	39.1	39.7	39.2	39.2	40.3	39.4	40	39.7	39.8	39.8	39	39.2	39.2
39.5	38.7	38.9	39.2	38.1	39.5	38.5	40.3	39.9	39.7	39.5	39	39.4	38.7
39.3	38.8	40	39.4	39	38.7	38.9	39.9	39.6	39.5	39.2	39	39.3	39
40	39.2	39.7	39.5	39.6	39.5	39.1	39.9	39.8	40	39.7	39.4	39.7	39.5
39.7	39.3	40.7	39.2	39.6	39.5	39.2	39.5	39.5	40	39.6	38.9	39.2	39
39.8	*9.4*	*39.3*	*39*	*39.5*	*39.3*	*39.6*	39.4	*39.7*	*39.3*	*39.8*	*39*	*39.7*	39
39.5	38.8	39.6	41.1	39.8	39.3	39.2	39.5	39.8	39.7	39.2	39.2	41.7	39.1
40.2	39.4	39.4	41.2	39.7	39.5	39.1	38.9	40.5	40	39.7	39.2	40.8	38.8
39.8	39.7	39.5	41.6	39.3	39.7	39.7	39.9	39.7	39.4	39.5	38.9	42	38.9
40.1	39.5	39.5	39.8	39.3	38.9	39.4	39.4	39.8	39.4	39.7	39 x		38.9
39.9	39	39.7 x	x		39	40	39.5	37.9	39.7	39.1	39.7		39
39.8	39.1	39.5			39	39.5	39.3	39.1	39.9	39.6	39		38.9
39.8	38.8	39.7			38.3	39.4	39.6	39.4	39.7	39.3	39.7		38.4
39.3	38.8	39.8			39	39.5	40	39.3	39.4	38.9	38.6		39
39.3	39.1	39.6			39.3	39.3	39.8	39.7	39.6	38.9	38.9		39
39.5	38.4	39.6			39.1	38.8	39.2	39.7	39.4	39.3	39		38.5
38.4	39.3	39.6			39	39.4	38.8	39.5	39.6	38.4	39		38.2
39.7	39.3	39.4			39.2	39	39.6	39.8	39.8	39.4	38.8		39
39.5	38.8	39.8			38.6	39.6	39.6	39.5	39.5	39.7	39.3		39.2
39.1	39.4	38.4			38.8	39.6	38.6	39.2	39	39	39.3		38.9
38.8	38.8	39.7			39.2	38.7	39.2	39.8	39.4	39.8	39		39.8
39.5	39.5	39.2			39.3	39.8	39	39.9	39.6	39	38.9		39
39.4	39.2	38.9			41.2	39.3	39.5	40	39.7	39.3	39		38.8
38.9	39	39.2			41.3	39.4	39.5	39.8	39.2	39.3	38.7		39
39.1	39	39.8			40.6	39.2	39.6	40.1	39.7	39.6	39		39
39.2	39	39.2			41	39	39.5	39.5	39.9	39.3	39.2		39.1
39.5	39.3	39.6			41.9	38.7	39.6	39.9	39.5	39.1	39.2		39
39.1	39	39.5			41.3	38.7	39.6	39.5	39.5	39.1	38.9		39
39.2	38.9	39.5			40.3	38.9	39.7	39.8	39.4	39.2	39		38.9
39.2	39.1	39.4			40.9	38.7	39.4	39	39.7	38.9	38.6		39.4
39.4	39.1	38.8			40.5	39.4	39.6	39.2	39.1	39.5	39		39.3
39.6	39	38.7			40.2	39.3	39.6	39.2	39.7	39.3	39.1		39.1
39.6	39.2	39.5			40.3	39.4	39.5	39.9	39.8	39.8	39.5		39.3
39.7	38.9	39.7			39.6	39	39.4	39.4	39.8	39.5	39.7		39
39	*38.8*	39.3			*38.8*	*39.7*	*39.6*	*39.7*	*40.1*	39.8	*38.8*		*39*
39.1	39.6	39.3			39.6	39.2	39.7	39.9	39.8	39.7	39.5		39.1
39.1	39.7	39.3			39	39.8	39.5	39.5	39.8	39.5	38.8		39.4
39.7	39.6	39.4			39.3	39.4	39.7	39.8	39.7	39.7	38.7		39.4
39.3	39.2	39.8			39.6	39.5	39.6	39.5	40.5	39.2	39		39.7
39.7	39.6	39.8			39.6	39	39.9	39.8	40.2	39.2	39.1		39.3
39.8	39	39.5			39.7	39.2	40	39.3	39.9	39.8	39.5		39
39.7	39.4	39.3			39.4	39.5	40	39.4	39.5	39.6	39.6		39.1
39.4	38.8	39.1			39.6	39.6	40.1	39.6	40.1	39.8	39.6		41.1
39	38.8	39.3			39.6	40.8	39.7	39.5	39.6	39.4	38.9		41.5
40.4	39.5	39.5			39.3	41.2	41	39.3	40.9	39.5	38.9		42
41	39.6	39.2			39.4	40	41.1	40.3	41.2	39.7	40.1	*	
41	39	39.4			39.5	41.9	41.4	41 *		39.5	39.8		
41.9	40	41.6			39.3	42	41.6	41.7		40.2	41.8		
*	40	41.5			39	41.3 *		41.6		40.4	41.5		
	39	40.6			39.1	40.6	*			41	41		
	39.3	41			39.3 *					41.6 *			
	39.2	41			39.2					39.5			
	39.3	40.4			39.2					39.3			
	39	39.8			39.1					39.1			
	39.2	39.5			39.7					38.8			
	39.8	39			39.1					38.8			
	39.5	39.5			39.7					39.8			
	40.1	40			39.5					39.9			
	39.6	40.3			39.8					39.9			
	39.7	39.6			39.7					39.8			
	39	39.5			39.2					39.7			
	38.7	40.6			39.3					39.8			
	39.2	40			39.4					39.7			
	38.6	38.6			38.7					38.8			
	38.8	38.9			38.4					38.5			

sh. 93	sh. 94	sh. 95	sh. 96	sh. 97	sh. 98	sh. 99	sh. 100	sh. 101	sh. 102	sh. 105	sh. 107	sh. 108	sh. 111
39.5	39	38.5	39.5	39.1	39.1	38.7	39.5	39.8	39.3	38.8	38	39	39.2
39.1	39.3	39.5	39.3	39.8	39.9	39.5	39.8	40.3	39.2	39.5	39.6	39.6	39.3
39.6	39.6	39.8	39.6	39.9	39.8	39	39.9	40.3	39.8	40	39.6	39.5	40
39.9	39	40	39.3	39.3	39.4	39.5	39.3	40	39.9	39.9	39.4	40.1	40
39.4	39.6	39.3	39.7	39.2	39.7	39.3	39.4	39.5	39.3	39.3	39.6	39.2	39.3
39.3	39.6	39.7	39.5	39.5	39.3	39.3	39.7	39.6	39.8	39.5	39.6	40.5	39.5
39.5	39.7	38.8	39	39.4	39.3	39.5	38.9	39	39	38.9	39	39.6	39.5
39.8	39.3	39.1	39.4	39.1	39.8	39.6	39.5	39.1	39.3	38.6	39	39.2	39.2
39.6	39.5	38.8	39.3	39.3	39.1	39.4	39.7	39.1	39	38.9	39.2	38.9	39
39.4	40.1	39.6	39.6	38.8	39.3	39.5	39.3	39.1	38.7	39.5	39.1	39	39.1
39.7	39.9	39.1	40	38.5	39.4	39.4	39.2	39.3	39.2	39.2	38.7	39	40
40	40	39.8	40	39.4	39.7	40.1	39.4	40.7	39.5	39.5	38.5	40.1	39.8
41.5	**40.6**	39.7	**40.7**	39.8	39.5	39.5	40.5	40	39.8	39.1	39.2	39.6	39.2
41.8	42	39.9	42	39.3	39.3	39.4	40.2	40.2	39.6	38.9	38.3	39	39.7
40.6	40.7	40.5	41.7	39.3	39.6	39.2	39.2	39.7	39	39.3	**40.3**	40	39.3
41.2	39.5	41.3	40.4	39.4	39.8	39.7	38.7	39.7	39.3	39.5	41.1	**40.7**	40
41.2	39.3	42	39.2	39.7	39.8	39.2	39.2	39.3	39.7	39.9	41.7	40.9	39.9
40.3	39.4	40.4	39.4	40.2	39.9	39.8	40.7	40.2	39.5	40	40.9	40.1	39.7
39.5	39.3	40.6	39.3	39.4	39.6	38.8	39.7	39.8	38.8	39.8	40.5	39.2	39.3
39	39.1	40.2	39.6	39.7	39.9	39.4	39.6	40.1	39.4	39.8	39.9	39.8	39.4
39	39.2	40	39.3	39.9	39.8	39.5	40.2	39.6	39.3	39.9	39.9	40.3	39.5
38.9	39.3	39.8	39.5	39.4	39.7	39.2	39.5	39.4	39	39.6	39.2	40	39.2
39.1	38.9	39.3	39.4	39.7	39.5	39.5	39.2	39.6	39.1	39.2	39	39	39
39.1	39.1	39.3	39.6	39.6	39.4	39.5	39.3	39.5	39.9	39.1	39.6	39.6	39
38.8	39	39.3	39.7	39.6	39.3	40.3	39.2	38.8	39.6	39.1	39.2	38.7	39.5
39.5	39.1	39.6	38.9	39.6	39.5	40.6	39.3	39.5	39.8	39.2	39.5	39.3	39.6
39	39.1	39.4	39.9	39.1	39.6	41.1	40	39.4	39.5	39.4	39.3	39.5	39
39	39.1	39.7	39.2	39.7	39.9	39.5	40.5	39.4	39	39.5	39.2	39.3	39.6
38.8	38.9	39.5	39	39.5	40.2	39	39.6	39.5	39.2	39.3	39.5	39.1	39
39.3	38.7	38.7	39.3	39	39.5	38.9	39.8	39.5	39.3	39.1	39	38.9	39.4
38.8	39.3	40	39	39.6	39.6	39	39.5	39.7	38.7	39.7	39.2	39.3	39.1
38.9	39.4	39.8	39.4	39.5	39.8	39	39.8	39.6	38.8	40.5	39.3	39.6	39.3
39.6	*39.2*	*39.7*	*39.6*	*39.6*	*40.2*	*39.5*	*39*	*40*	*39.9*	*40.3*	*39.4*	*39.7*	*39.7*
39.4	39.5	38.8	39.4	39	40	39.7	39.9	39.7	39.3	39.7	39.3	39.7	39.7
39.6	39.2	38.5	39.5	39	39.8	39.5	39.5	39	39	39.6	39.6	39.4	39.8
39.7	39.3	39.3	39.3	38.9	39	38.8	39.7	39.5	39.6	39.4	39.6	38.8	38.7
39.1	39.6	39.2	39	38.6	38.8	39.1	39.2	39.2	39	39.7	39.3	38.7	39.2
38.7	38.8	39.4	39	39	39.7	39.2	39.2	39.2	39.3	39.8	38.5	39.1	39.1
39	39.1	39.9	39.3	38.8	39.5	40.3	39.4	39.1	39.9	39.2	39	39.7	38.9
38.6	38.8	38.5	38.9	38.2	38.9	39.1	38.9	38.8	38.5	38.9	38	38.8	38.8
39	39.4	39.2	39	39.6	39	39.2	39.5	39	39.3	39.2	38.8	38.6	39.9
39.2	40.1	39.6	39.2	39.6	39.8	39.8	39	39.8	39.6	39.1	39.1	39.3	39.9
39.3	40.9	39.4	38.5	39	39.6	39.4	39.4	39.5	39.2	39.6	38.9	39.9	40.1
39.3	*40.8*	*39.5*	*39.5*	*39.7*	*39.2*	*39.2*	*39.3*	*39.7*	*39*	*39.4*	*39.2*	*39*	*40*
39	40.5	39.3	39	39.8	39.6	39.5	39.5	39.7	39.5	39	39.5	39.3	40
39.4	39.7	39.8	39.2	39.4	39.5	39.5	39.4	39.6	39.2	38.5	39.4	38.9	39.5
39.9	39	39	38.6	40.3	39.1	39	39.4	39.4	38.5	39.2	39.2	39.4	40
39.1	39.2	39.8	39.3	39.5	39.4	39.1	39.8	39	39.1	38.9	38.6	39	40.3
39.4	39.7	39.3	39	39.7	39	39.2	38.7	39.3	39	39.3	38.7	39.4	40
39.8	39.4	39	38.9	39.3	39.3	39	39	39.1	38.6	39.4	39.2	39.2	39.8
39	39.6	39.7	39	39.6	38.5	39.2	39.5	39	39.3	38.6	38.7	39.5	39.5
39.3	39.7	39.8	39.1	39.4	39	39	39.1	39.6	39	39.3	38	39.4	39.6
38.9	39.4	39.3	38.9	39.7	39.7	39.5	39.7	39.5	39.1	39.5	39.4	39.4	38.9
38.9	39.4	38.7	39	39.4	39.5	39.4	39.6	39.6	39.1	38.7	39	39.2	39.1
38.4	39.7	38.5	38.9	38.6	39.1	39.5	38.9	39.5	39	38.5	38.9	38.6	39
38.9	39.7	39.7	39.1	39.2	39	39.6	39.6	39.4	39.2	39.2	39	39.4	39
39.1	39.3	39.2	39.3	39.2	38.8	39.6	39.4	39.6	39	39.1	39	39.2	38.9
39	39.7	39	39.5	38.9	38.9	39.2	39.3	39.2	38.9	39.4	38.9	39	38.5
38.9	39.6	39.4	39	39.4	39.3	39.5	39.2	39.4	39.7	39.3	39	38.8	39
38.5	38.9	39.1	39.8	39	38.8	39.2	39	39.3	38.9	39.9	39.3	38.2	39
39	39.5	38.9	39.3	39.4	38.6	38.6	39.6	39.4	39.6	39.4	39.2	38.5	39
38.6	39	39.5	39.6	39	39	38.7	39.4	39.3	39.6	39.3	38.9	39.2	39.2
39.2	39.2	39.2	39.3	38.8	39.1	39.1	39.7	39.7	39.6	39.3	38.7	39.2	39.1
38.9	39.3	39	39	39.3	39.3	38.9	38.6	38.9	39.6	39.2	39.6	39.7	39
39.3	39.2	39.8	39.6	38.9	39.6	39.4	39.7	39.6	39.6	39.1	39.4	39.3	39.1
38.9	39.1	39.2	39	39.1	39.2	39.5	39.1	39.5	39.3	38.8	39.1	38.8	39
38.7	39	39.3	39.3	39.4	39	38.7	39	39	39.2	39	39	39.2	39.2
39	39.4	38.8	38.7	38.8	38.8	39.5	39.7	39.6	39.4	39.2	39	38.6	39.2
39.3	39.2	39.5	39.5	39	39.1	39.5	39.5	39.6	39.3	39	39.4	39.5	39.1
38.9	39.3	39.1	39.4	39.6	39	39	39.7	39.4	39.6	39	39.6	39.6	39
39.2	40.3	39.8	39.3	39.3	39.6	39	39.8	39.7	39.4	39.4	39.6	39.8	39.2
39	39.8	39.5	38.8	39	39.5	38.9	39.4	39.5	38.6	39.6	39.8	39.4	39.5
39.3	***39.4***	***39.2***	***39***	***39.2***	***39.7***	***39***	***39***	***39.3***	***39.1***	***38.9***	***39***	***39.4***	***39.4***
38.8	39.5	39.2	39.1	39.3	39.5	39.3	39.9	39.3	39.2	38.7	38.7	39.7	39.2
39.4	39.1	39	39.2	39.5	39.2	39.2	39.6	39.5	39.4	39	39.6	39.6	39.4
39	40.1	39.6	39.3	39	38.7	39.3	39.2	39.9	39.5	39.2	39.3	39.6	39.2
39.5	39.7	39.5	39.4	39.5	39.5	39.9	39.3	39.9	39.5	39	39.8	39	39.3
39.5	39.4	39.4	39.5	39.2	39.1	39.7	3836	39.9	38.5	38.8	39.2	39.5	39.2
39	39	39.2	39.6	38.8	39.3	38.9	38.6	39.1	39.4	39.2	39.6	39.2	39.1
38.7	39.4	39.2	39.6	39	39.7	39	38.7	38.9	39.4	39	39.5	39.1	39.1
38.8	39.6	38.7	39.3	39.2	39.7	39	39.1	39.3	39.7	38.9	40.1	39	39.1
39.2	39	39.5	39	39.7	39.9	39.1	40.8	39.3	40.6	39	40.4	39	39.3
39.5	39.4	40.6	39.3	39.5	41.2	39	41.6	39.6	41.6	39.6	41.5	40.5	39.7
39.7	39.5	41.3	39.6	39.4	41.9	39.6	41.3	39.2	41.5	39.7 nd		41.5	39.5
41.5	38.9	41.2	38.6	39.7	42	38.3	40.8	39.2	41.2	39.1 *		40.6	39.7
41.9	39.8	41	39.4	39.1	41.5	39.3	42	39.7	40.1	39.5		42	39.5
40.7	39.6 *		39.3	39.6	41.7	39.5	41.7	39.2 *		39.1		41.2	39.6
*	39.2		38.8	39.5 *		39 *		38.7		39		40.3	39.4
	38.7		38.8	40.1		39.1		38.8		39.3	*		39.5
	39		38.8	39.6		38.7		39.4		39.1			39.7
	39.3		39.3	39.9		39.3		39		39.3			39.6
	39.2		39.4	41.4		39.1		39.4		39.5			39.6
	39.2		39.3	40.5		39.3		39.5		39.3			39.4
	38.8		39.2	41.4		39		39.4		39.1			39.5
	39.8		39.3	41.5		39.2		39.6		40.1			39.7
	40		40	41.2		39.8		40.1		39.2			39.6
	40.2		39.8	41		40		39.7		39.1			39.6
	39.7		38.7	40.7		39.5		39.5		38.8			39.4
	39.2		38.7	39.7		39.1		39.1		39			39.2
	38.9		38.9	39.3		39.3		39.5		39.3			39.1
	39.1		39.2	39.2		39		39		39.2			38.9
	38.7		38.5	38.5		38.8		39		38.8			38.5
	38.6		38.5	38.9		38.5		38.9		39			38.6

sh. 112	sh. 113	sh. 115
39.6	39.8	39.3
39.2	39.5	39.5
40.1	39.5	39.9
40	39.6	39.4
39.7	39.4	39.9
39.5	39.7	39.6
39.7	39.2	39.9
39.5	39.5	39.2
39.4	39.6	39.3
39.3	39.8	39.3
39.1	**40.5**	39
39.8	40.8	39.4
39	42	39.5
39.5	41.3	39.4
39.7	41.3	39.3
40.6	39.9	41.7
40.4	38.9	41.9
41.2	39.3	40.8
41.6	39.2	41.3
40.3	39.7	40.3
39.8	39.8	39.4
40.1	39.6	39.4
39.4	39.5	39.2
39.6	39.3	39
39.3	39.1	39.1
39.4	39.4	39.3
39.5	39	38.8
39.6	39.7	39.1
40	39.3	39.2
39.5	39.4	38.7
39.2	39.4	39.1
39.8	38.6	39.6
39.3	*39.8*	*39.2*
39.4	39.7	39.4
39.5	39.9	39.2
39.8	39	38.8
39.1	39.2	39.2
39.7	39	39.3
39.6	39.2	38.9
38.4	39.7	39.2
39	40.2	39.3
39.8	40	40.2
39.7	40	40
39.4	*40*	*39.5*
39.5	39.8	39.7
39	39.8	39
39.4	39.1	39.3
39.9	39.5	38.7
38.5	37.9	39.5
39	39.2	39.4
39.6	39.3	39.3
39.6	39.5	39
40	38.9	39.6
39.7	39.5	38.4
39.6	38.7	39.1
39.9	39.2	39.6
39.5	39.1	39.9
39	38.8	39.3
39.2	38.6	39.5
39	39.6	39
39.2	38.6	39.7
39	39.2	39.6
39.7	39.7	40.5
39.7	39.3	39.6
39.3	39.9	39.9
39.2	39.2	39.5
39.4	39	39.7
39.4	39.3	38.8
39.2	39.3	39.5
39.2	39.8	39.4
39.4	39.6	39.5
39.1	39.4	39.7
39.9	***38.8***	***38.9***
40	39	38.9
39.5	40.1	39
40	40.5	39.7
40.4	40.4	39.7
39.8	39.9	39.8
39.6	39.4	39.2
39.5	39.4	39.2
40.1	39.8	39.8
39.6	40	39.6
40.9	41	40.8
41.6	41	41.3
41.8 *		41.6
41.8		
41		
40.6		

*

Appendix 5.7 Temperature record of sheep in the second experiment

Day	Date	sh. 116	sh. 117	sh. 118	sh. 119	sh. 120	sh. 121	sh. 122	sh. 123	sh. 124	sh. 125	sh. 126	sh. 127	sh. 128
0	2/23/96	39.7	39.2	39.3	39.3	38.6	38.7	38.6	39.2	39.5	38.8	39.1	39	38.7
1	2/24/96	39.5	39.4	39	41.4	38.7	39.1	38.9	38.5	38.8	38.5	38.3	39.1	38.8
2	2/25/96	39.2	39.1	38.6	39.8	39	39.1	39.3	38.9	39	38.7	38.8	38.7	38.7
3	2/26/96	39	39.6	39	40.5	39.5	39.1	39.1	39.2	39.1	39.4	39.3	39.4	39.3
4	2/27/96	38.9	39.2	38.7	38.9	38.7	39.2	39.1	38.7	39.3	38.9	39	39.4	39
5	2/28/96	39.4	39.2	39.1	38.3	39.1	39.1	39.5	39	38.7	39	38.9	38.9	38.4
6	2/29/96	38.5	39.1	39	38.7	39	38.8	39.5	38.6	39.1	39.5	38.7	39	38.7
7	3/1/96	37.9	39.1	38.9	39.1	39.3	39.4	39.2	39	39.3	38.5	39	39	38.1
8	3/2/96	38.9	39.1	38.9	39.4	38.1	39.9	40.1	40.5	39.1	39.3	39.1	39.1	38.5
9	3/3/96	39.2	39.8	41	39.4	39.4	40.6	40.3	40.7	39	39.2	41	39.4	41.1
10	3/4/96	38.8	41	41.3	39	39	41.8	40.6	41.1	39.2	41.1	41.6	38.9	40.7
11	3/5/96	38.9	40.8	40.5	38.8	38.9	41.4 *		41.1	39.2 *		40.7	39.6	39.9
12	3/6/96	38.6	41.3	40.4	38.5	39.5	40.3		40	39.2		40.1	38.8	39.5
13	3/7/96	39	41.2	40	39	39.2	39.2		39.4	39.4		39.5	39.5	39.4
14	3/8/96	395	40.7	39.5	39.9	39.6	28.7		39.6	39		38.9	39.2	40
15	3/9/96	39.3	39.8	39	38.7	39.6	38.4		39.1	38.7		38.7	39.1	38.1
16	3/10/96	38.9	39	38.8	38.9	39.4	39		39	38.7		38.9	39.1	38.7
17	3/11/96	39	38.6	38.3	38.8	40.2	39.2		38.8	38.8		38.7	39.1	38.8
18	3/12/96	39.8	38.7	39.3	39.3	39.5	39.8		39	39.5		38.6	39.4	39.2
19	3/13/96	39	38.4	39.3	39.1	39.2	38.8		38.9	39		38.7	38.8	39.2
20	3/14/96	38.2	38.1	39.3	38.6	39.5	38.7		38.5	39.5		38.9	39.1	39.4
21	3/15/96	39.1	39	38.7	38.7	40	38.7		38.7	39.4		38.8	39.5	39
22	3/16/96	39	38.9	38.8	38.8	39.5	39		38.9	39.5		39	39.6	39.1
23	3/17/96	39	38.5	39	39.1	40.1	39.1		38.6	39.3		39.2	39.4	39.3
24	3/18/96	39.2	38.5	39.1	39	39.5	41.2		38.4	39.6		40.2	39.2	38.7
25	3/19/96	39.1	39.3	38.8	39	39.5	40		38.3	39.3		39.4	39.8	39
26	3/20/96	39.3	38.7	39.4	39	39.6	39.6		39.2	39.3		39.6	39.6	39.1
27	3/21/96	39.4	38.9	39	39.3	39.1	39.2		38.6	39.6		39.2	39.7	38.7
28	3/22/96	39.1	39.2	39.5	39.5	39.6	39.6		38.7	39.6		39.4	39.4	39
29	3/23/96	39.3	39.5	39.3	39.2	39.6	39.6		39.6	39		39.2	39.6	39.2
30	3/24/96	39	39.4	38.9	39.1	38.9	39.2		38.8	39		39.1	39.2	39.6
31	3/25/96	38.8	39	38.9	38.8	38.7	39.1		38.6	39.1		38.9	39	38.7
32	3/26/96	38.8	39.2	38.8	39	39.1	39.1		38.9	39.5		39	39.4	38.8
33	3/27/96	39.3	39.1	39.1	38.9	39.5	39.2		38.5	39.6		39.2	39.4	38.9
34	3/28/96	39	38.9	39	39	39	39		38.8	39.2		38.8	39.3	39.2
35	3/29/96	39.2	38.9	39.2	38.6	39.3	39		38.9	39		39.2	39.3	39.1
36	3/30/96	39	38.8	39	38.9	39.1	39		39	39.1		39	38.4	38.5
37	3/31/96	38.8	38.6	39.2	39	39.1	38.7		39.2	39		39	38.6	38.8
38	4/1/96	39	38.9	38.5	39.1	39	38.9		38.5	39.1		39.1	39.1	39.2
39	4/2/96	39	39.1	38.8	38.9	39.2	39		38.6	39.1		39	39.2	38.8
40	4/3/96	39.1	38.8	39	39	39.3	39.2		38.7	39.3		38.7	39.1	38.8
41	*4/4/96*	*38.9*	*38.7*	*38.8*	*39.1*	*39.2*	*39.1*		*38.9*	*39.3*		*38.8*	*39*	*39.2*
42	4/5/96	38.8	39	39.3	39.2	39.5	39		38.7	39.2		39.2	39	38.6
43	4/6/96	38.6	39.2	39.3	39.2	39.4	39.5		38.8	39.1		39	39.2	38.5
44	4/7/96	38.9	39.3	39.2	38.9	39.6	39		38.9	39.2		39.1	39.4	38.5
45	4/8/96	38.8	39.2	39.9	38.8	39.3	39		38.8	38.8		38.5	39.2	38.5
46	4/9/96	39	39.5	39	39.4	39.3	39.2		39	39.1		38.8	39.1	39
47	4/10/96	38.7	39.2	39	39.4	39.1	39.2		38.9	38.9		39	38.9	39.1
48	4/11/96	39	39.5	39.2	39.6	39.6	39.3		40.2	39		38.7	39.8	38.5
49	4/12/96	39.2	39.4	39.1	39.2	39.1	39.2		39.8	39.2		38.8	39.6	39.5
50	4/13/96	40.4	39.6	38.8	38.7	39.6	39.1		39.1	40.5		38.8	40.3	38.6
51	4/14/96	41.4	39.4	38.8	39.1	39.5	39.6		39.2	41.1		38.6	41	39
52	4/15/96	41.4	39	38.5	38.8	39.3	39.6		38.6 *			38.8	41.1	39.1
53	4/16/96	40.7	39.6	39.1	39.5	39.9	39.7		38.7			39.1	41.4	39.1
54	4/17/96 *		39.1	38.8	38.8	39.3	40.2		38.6			38.5	41.7	38.4
55	4/18/96		39.4	38.9	39.3	39.1	39.2		38.8			39	41.1	38.5
56	4/19/96		38.9	39.4	38.8	39.7	39.3		38.8			39.3	41	38.8
57	4/20/96		39	39.2	39	39.5	39		38.7			39.2 *		39
58	4/21/96		39.7	39.4	39.3	39.4	39.4		39.2			40		38.6
59	4/22/96		39.2	38.7	39	39.7	39		38.6			41.1		38.9
60	4/23/96		39.1	38.8	39.1	39	39		38.8			41.2		39.5
61	4/24/96		39.2	39.1	38.8	39	38.7		38.8			40.6		39.3
62	4/25/96		39.1	38.9	38.8	39	38.6		39.1			40		39.2
63	*4/26/96*		*39.3*	*39.1*	*38.6*	*39.1*	*38.8*		*38.9*			*39.6*		*39.3*
64	4/27/96		39.6	41.7	39.6	39.2	39.2		38.9			38.9		38.8
65	4/28/96		39.6	41.8	38.9	39.1	39.1		38.7			38.9		38.6
66	4/29/96		39.7	41.6	39.4	39.1	39.1		38.9			39		39.1
67	4/30/96		39.5	40.6	39.4	38.9	39		39.1			39.5		39
68	5/1/96		40	40	39.1	39.6	39.4		38.9			39.6		39
69	5/2/96		39.1	39.6	39	39.4	39.3		39			39.5		38.8
70	5/3/96		39.4	39.2	40.2	39.7	38.8		38.7			39.7		38.6
71	5/4/96		39.4	39.5	40.7	39.4	39.2		39			39		38.8
72	5/5/96		39.7	39.3	41.3	39.2	39.4		39.1			39		39.4
73	5/6/96		39.3	39.3	41.9	39.9	38.9		38.8			39		38.8
74	5/7/96		39.3	39.5	40.3	39.7	39		38.8			39.3		38.6
75	5/8/96		39.3	39.1	40.8	39	38.8		38.8			39.2		39.1
76	5/9/96		40.8	39.1 *		39.4	39.4		38.7			39.3		38.7
77	5/10/96		39.8	39.5		39.4	39.6		39			39		38.9
78	5/11/96		39.1	39.5		39.7	39.2		39.4			39.1		38.8
79	5/12/96		40.5	39.5		39.8	39.7		38.7			39.1		38.8
80	5/13/96		38.9	39.3		40.3	39.3		39			39.1		38.7
81	5/14/96		38.5	39.1		40.2	39.4		38.8			39		38.8
82	5/15/96		38.5	39.4		40	39.4		39.5			39.1		39.1
83	5/16/96		39.1	39.3		39.3	40		39.2			38.7		39.2
84	5/17/96		39	39.1		39	40		40.4			38.8		38.6
85	5/18/96		39.3	39.2		39.5	39.9		39.7			38.8		39.6
86	5/19/96		39.3	39.1		39.3	39.3		39.1			38.8		38.3
87	5/20/96		38.9	39.6		38.8	39.4		39.7			3.7		38.5
88	5/21/96		38.6	39.4		39.5	39.4		39			39		38.6
89	5/22/96		39	39.5		39.3	39.3		38.8			39.1		38.9
90	5/23/96		38.9	3.5		39.9	39.4		39.1			38.8		38.8
91	5/24/96		39.2	39.6		39.7	39.1		38.9			39		39.3

sh. 129	sh. 131	sh. 132	sh. 133	sh. 134	sh. 135	sh. 136	sh. 137	sh. 138	sh. 139	sh. 140	sh. 141	sh. 142	sh. 143	sh. 144
39.5	39.2	39.2	39.6	39	39.1	39.5	39.6	39.4	38.7	39.5	38.7	38.8	39	39.6
38.7	39.1	39.7	39.4	38.6	39.4	38.8	39.6	39.4	39	39	38.7	39.1	39.5	39.2
39.9	39.5	39.6	39.5	38.4	39.5	38.7	38.9	38.8	39.2	39.2	39.1	38.8	39.4	39
39.1	39.3	39.5	39.1	39.3	39.7	39.2	39	38.6	39	39.4	39.2	39.3	39.2	39.1
39.2	38.5	38.6	39.2	37.8	39.2	39	38.8	38.9	39	39.1	38.6	39.1	39.1	39.2
39	38.4	39.2	39	38.7	39.1	39	38.8	38.3	39	39.1	38.2	39.3	39	39.3
39.1	39.1	38.7	38.9	38.5	38.9	39.1	38.7	38.5	38.9	39.1	38.8	39.1	39.1	39.3
39.7	38.7	38.5	39.5	39.4	39.6	39.3	39	38.6	39	39.2	38.7	38.9	39.1	39.9
39	38.9	39.4	39.3	40.4	41.3	39.7	39.2	38.9	40.2	39.3	38.1	39.6	41.1	40.3
38.6	38.6	39.7	39.9	41.6	41.6	41.6	38.9	39.2	41.3	39.3	38.9	39	41.8	40.2
38.3	39.3	39.6	40.8	40.7	41.1 *		**39.3**	40.4	40.7	39.3	38.8	41.1	40.9	40.4
39.1	39	**38.9**	41.1 *		40.7			41.3	40.5	39.3	**39.1**	40.9	41.4	39.6
39.4	38.6		40.1		39.7			40.7	40.5	39.6	38.7	41	40.4	39.5
39.5	39.4	*			39.3			40.8	38.9	39.5		40.8	39.6	40
39.6	38.8				39.3			40.8	39.2	39.4		39.6	40.5	40.6
39.2	39.1				38.6			39.3	38.8	39		39.8	39	39.3
39	39.2				39.2			38.8	38.7	39.2		39.7	39.3	39
39	39				38.8			38.7	38.7	39		39.5	39.2	38.7
38.3	38.9				39.3			38.6	38.8	39.5		39.6	39.2	39.1
39	39.1				39			39.2	39.3	39.5		38.5	39	39.3
38.9	39.2				39.3			38.2	38.1	39.2		39	39.1	38.9
39.7	39.2				39.3			39.1	38.7	39.3		39.6	39.1	38.8
39.4	39.2				39.2			39	38.9	39.3		39.5	39	38.9
39.2	39.1				39			39.2	39.1	39.2		39.2	39	39.1
38	38.9				39.2			38.9	38.9	39.2		38.2	39.1	38.9
39.2	39.4				39.4			38.8	39	39.2		39.9	39.2	39.3
40	39.1				39.5			39	39	39.5		39.2	39.8	39.5
39.6	39				39.8			39.2	38.8	39.7		39.3	39.5	39.5
39.5	39.5				39.2			39.3	38.9	40		39.8	39.2	38.8
39.7	39.2				40			39.7	39	39.6		39.7	39.5	39.7
38.9	38.7				39.4			39	38.6	39.4		39.1	39.1	38.7
39.1	39.1				39			38.6	38.6	39		39	38.7	38.9
39.2	39.2				39.3			39.3	39	39.1		39	39	38.9
38.8	38.9				38.8			38.5	39	38.5		39.1	39.1	38.8
38.9	39.2				39.2			39	39	39.3		38.8	38.6	39
39.2	38.9				39.3			39	39.1	39.1		38.7	38.8	39.5
38.8	38.9				39.1			38.8	38.8	39.2		39	39.4	38.8
39.1	38.9				39			38.9	38.6	39		39.2	39.5	39
38.7	39				39.1			39.1	38.8	39.2		39	39.1	38.6
38.7	39.1				39.1			39.2	39	39.3		39.1	39	38.9
38.6	39.3				39.1			39.2	39	39.2		39	39.1	38.9
38.9	*39*				*39.3*			*39*	*39.1*	*39.3*		*39.2*	*39.1*	*39.1*
39.4	39.2				39			38.9	39	39.3		39.1	39	38.8
39.1	39.2				39.1			38.7	38.7	38.9		39.2	38.6	38.7
39.5	38.9				39.2			38.5	38.9	39.3		39.4	39	38.6
38.8	38.8				39.2			38.6	38.7	39.3		39.5	38.7	38.7
39.2	39.5				39.1			38.8	38.6	39		39.1	38.9	38.8
39.3	38.8				39.5			38.9	39.2	39.2		39.2	38.9	38.7
39.9	39.3				39.4			39.3	39	39.8		39.2	39.4	38.8
39.3	38.9				39.8			39.2	39.2	39.5		39	39.5	39.4
39	38.8				39.2			39	39.3	39.4		39.2	39.5	39.1
38.8	38.6				39.8			38.4	39	39.2		39.3	39.3	39
39	38.6				39.2			38.8	38.6	39.2		39.3	39.2	39.4
39	38.9				39.9			38.8	39.2	39.3		39.1	38.5	39.4
39.4	39.1				39.5			38.6	38.5	38.8		39.5	38.7	3.6
39.4	39				39.6			38.8	39.2	39.2		39.3	38.9	39.4
39	39.2				38.8			39.3	39.3	39.4		39.5	39	39
39.1	38.6				39			39.3	38.7	38.9		39.6	39	39
39.3	39.3				39.4			39.2	38.4	39.1		39.2	38.8	39.1
39.1	39.1				39			39	38.8	38.6		39.3	38.9	38.9
39.2	38.6				39.2			39.3	38.9	39.1		39	38.9	38.9
39	38.2				39.3			39.2	39.2	39		39	38.7	39
38.9	38.6				39.2			38.7	39.3	38.7		39	38.9	38.8
39.2	*38.5*				*39.1*			*39.2*	*39.1*	*38.7*		*39.1*	*39.3*	*39.2*
39.2	39.2				39.2			38.8	39.2	39.4		39.6	38.9	39
39.4	39.2				39.2			39.3	38.7	39.1		39.2	39.5	39.2
39	38.9				39.2			39.1	38.9	39.3		39.7	39.4	39
38.8	39.2				39			39	38.9	39.2		39.5	39.6	38.8
39.5	39.5				39.5			39.3	39.4	39.2		38.8	39	39.2
39.3	39.5				39.4			39.3	39.6	39.1		38.9	39.2	39.3
39.4	40.5				39.2			38.9	39.5	39.1		39.2	39	38.8
38.6	38.9				39.8			39	39.1	39		39.4	39	39.2
39.4	39.3				39.5			38.8	39.2	39.3		39.4	39.2	39
39.2	41.1				39.1			38.7	39.3	39.9		38.8	39.1	38.9
38.9	40.8				39.2			39.2	39.2	39.7		39.2	39.3	38.6
39.4	41.3				39.4			38.8	38.9	41.6		38.8	39.2	38.8
39.8 *					39.6			38.9	39	41.1		39.5	39	40
40.1					40.5			39.1	39.3	40.6		39.5	39.3	40.6
40.6					39.5			39	38.5 *			38.9	39.4	41.6
41.2					39.5			38.5	39.3			38.9	39.3	41.8
41.5					41.5			38.8	38.7			38.8	39.5	41.4
41.1					40			38.8	38.9			39	39	40.2
41.6					41			38.7	39			39.3	39.5	39.3
41.4					40			38.9	38.9			39.2	39	38.7
40.4					40.1			39.3	38.8			39	38.8	38.5
*					39.7			39.5	39.6			39.2	38.9	38.8
					39.6			39.2	38.8			39.3	39.6	38.9
					39.8			39.2	39.2			38.8	39.1	38.4
					39.5			39	38.8			39.2	39.3	38.8
					39.6			39.1	39.3			39.2	39.6	39
					39.9			39.3	39			39	39.3	39
					39.6			38.9	39.3			39.3	39.1	39.2

sh. 145	sh. 146	sh. 147	sh. 148	sh. 149	sh. 150	sh. 151	sh. 152	sh. 153	sh. 154	sh. 155	sh. 156	sh. 157	sh. 158	sh. 159
39.5	39.3	39.3	39.5	39.3	39	39.6	39.2	39.3	39	39.3	38.7	39.3	39.1	39.6
39.3	39	40.5	39.2	39.4	39.3	39.5	38.5	39	38.7	39.1	39.4	39	38.5	39.2
39.5	39.5	39.4	39.2	39	39.5	38.9	39.1	38.6	39	39.4	38.9	39.3	38.9	39.2
39.1	39.5	39.3	39.1	39.1	39.6	39.1	39.2	39	38.8	38.9	39.5	39.6	39.9	39.3
39.7	39.1	38.6	39.1	39.2	39.6	38.7	38.8	38.7	38.9	39.3	38.6	39.3	39.6	39.5
39.1	39.3	38.3	39	39.1	38.3	38.5	38.8	39	38.4	39.7	38.2	39.4	39.2	38.6
38.9	39.1	39.4	38.2	38.2	38.9	38.7	39.1	38.6	38.9	39.1	39	39.7	38.8	38.3
39.4	38.8	39.3	39	38.7	39.2	38.8	38.8	38.6	39.4	38.9	38.7	39.2	39.1	39
40	40.3	39	39.2	40.8	40.5	40	38.8	39.5	38.4	39.4	40	39.7	40.2	40.7
41.3	40.2	40.8	39.3	41.4	41.2	41.3	38.8	39	41	39	41.4	40.5	40.5	41.6
41.9	41.4	41.2	40.8	40.9	41.9	41.6	38.9	**38.8**	41.5	39.6 *		41.3	40.8	40.8
*	41.7 *		41.5	40.1	40.5 *		38.9		40.7	39.3		40.8	40.4	41.1
	41		40.2	39.5	39.3		39		39.3	39.1		40.1	39.6	39.7
	39.5		41	39.5	38.9		39		39.2	39.2		39.4	39.5	39.7
	38.7		39	39.2	38.8		39.1		39.4	39.7		39.4	39.5	39.5
	38.8		38.9	39	38.9		38.7		39.8	38.8		38.7	39.6	39.5
	38.6		39.1	39.3	39		38.7		39.9	39.3		39	39	39.3
	39.2		38.9	38.8	38.9		38.7		39.2	38.3		38.8	39.1	39.6
	39.2		39.1	39.1	39.5		38.8		39.5	39.1		39	39.5	39.5
	38.8		38.6	38.8	39.1		39		38.9	39		39.3	39	39.3
	39.1		39	39.2	38.4		39.1		39	38.9		38.6	39.5	39.2
	39.3		39.2	39.4	38.9		38.7		39.2	39.6		39.4	39.6	39.1
	39.4		39	39	39		38.8		39	39		39.3	39.4	39
	39.3		39.1	38.8	39.3		38.7		39.1	39.2		39.2	39.5	39.2
	39.2		39.3	38.7	39.1		39.1		39	38.9		39.4	39.1	39.2
	39.2		39.5	39.1	39.2		39.5		39.3	39.2		39.3	39.8	39.8
	39.4		39.3	39.7	39.7		40.1		39.2	39.2		39.7	39.4	40.1
	39.4		39.4	39.7	39.6		39.7		39.1	39.6		39.7	39	40
	39.3		39.9	39.9	39.8		40.2		39.5	39.3		39.6	39.4	40.2
	39.5		39.6	39.9	39.6		39.6		39.3	39.9		39.9	38.9	39.6
	39.7		39.3	39	3.2		38.9		38.9	39.3		39.2	39	39.2
	38.9		38.8	39	39		38.9		38.5	38.8		39.2	38.9	39.1
	39		39	39.2	39		39		38.7	39.3		39.2	39	39.5
	39		39.2	39	39.4		38.8		38.9	39.2		39.4	39.1	39.1
	39		39	39.4	39		39		38.8	39.1		39.3	39.3	39.6
	39.1		39.2	39.5	39.2		39		38.7	39		39	39.3	39.5
	39.2		38.5	39.1	38.6		39		39.2	38.8		38.8	39.4	395
	39.1		38.8	39	38.4		39		39.2	39.1		39	39.3	39.3
	39.1		38.7	39.3	38.8		38.8		38.4	38.7		39.3	39	39
	39.1		38.8	39.2	39		38.8		38.7	38.7		39.3	39	38.8
	38.8		39	39.2	38.9		39		38.9	38.6		39.2	39	39
	39		*38.8*	*38.9*	*38.7*		*38.8*		*39*	*38.8*		*39*	*39.1*	*39.1*
	39		39.3	39.3	39		38.8		40.1	38.8		38.9	40.3	39
	39.2		39.5	39.2	38.7		38.7		39.9	38.8		38.9	40.1	39.1
	39.2		39.2	39.3	38.5		38.9		39.5	38.6		39.4	39.4	39
	39		39.3	9.4	38.5		38.7		39.3	39		39.4	38.8	39.1
	39.2		40.3	39.3	38.9		39		38.9	39		38.9	39.2	39.3
	38.9		39.7	39.2	38.7		38.2		39	39		39	39.1	39.5
	38.9		39	39.3	39.2		38.7		38.9	39.3		39.7	39	39.2
	39.1		39.7	39.6	39.3		39		39	39.4		39.2	39.2	39.5
	38.9		39.1	39.5	39.5		39.1		39	39		39.3	39.1	39
	39		38.8	39.3	39.3		39.2		39.4	39		39	38.9	39
	38.9		39	39.5	38.8		38.5		39.1	39.2		39.2	38.7	38.8
	39.2		39.2	39.3	39.5		39.1		39.2	39.4		39.6	39.3	39.2
	39.1		39	38.8	39.2		39.1		39.6	39.5		38.3	39.5	38.9
	39.5		39.2	39	39.4		38.8		39.5	39.1		39.3	39.3	38.8
	38.7		39.3	39.7	39.4		39.4		40.5	39.3		39.7	39.1	39.1
	39.4		38.8	39.4	39.2		39		40.6	39.2		40.6	39	39.2
	38.9		39.1	39.1	39.7		38.8		41.3	39.6		40.1	39.1	38.9
	39		39.2	38.4	39.6		38.7		39.5	39.4		39.9	39	39
	39.2		38.6	39.2	39.4		38.6		39.5	39		39.3	38.8	39
	39.3		38.6	39.1	39.4		38.8		39.3	39.2		39.5	38.9	39
	39.2		38.8	39.1	39.5		39		39.2	39.2		39.6	39	39
	38.7		*39.1*	*39.3*	*39.5*		*39.2*		*39.1*	*38.9*		*39.4*	*39.1*	*38.8*
	40		39.3	39.4	38.8		38.9		38.8	39		38.8	39	39.4
	38.8		39.2	39	39.2		39		39.2	39.6		39.1	39.1	38.7
	38.7		39.1	38.9	39.4		39.1		39.2	39.2		39.1	39.2	39.4
	38.9		39.2	39	39.4		39.2		39.2	39.2		39	39.3	39.5
	39.6		39.6	39.4	39.5		39.2		38.9	39.7		39.2	38.8	39.5
	38.7		39.4	39.2	39		39		38.9	39.5		39.3	38.8	39.5
	38.9		39.1	39.4	39.1		39.1		38.6	39.4		38.3	38.6	38.8
	39.1		39.1	39.5	39.2		39		39.4	39.1		39.5	39.5	38.9
	39.2		39.4	39.4	39.7		39		39	39.2		39.5	39.5	39.3
	38.8		39.1	39.3	39.2		40.2		39.4	40.8		3.2	39.1	39
	38.8		39.3	39.5	38.8		40.6		38.9	40.2		39.4	39.2	39
	39.2		39	40.3	39.2		41.1		38.5	40.6		39.3	39	38.7
	39.7		39.1	40.6	39.7		41.9		39.3	41.7		38.7	38.8	39
	39.6		39.5	41.8	40.9	*			39.5 *			39.3	38.8	39.1
	39.4		39	41.6	41.2				39			39.4	38.6	38.6
	39.2		38.7	41.2	41.5				38.5			39.3	38.9	39.3
	39.2		39.3	40.8	41.2				38.6			39.5	39.2	38.9
	39		39.1	40	41.3				39.1			39.1	39.1	39
	39.1		39.3	39.3	40.6				39.2			39.6	39.3	39.5
	39		39.4	39.1	40.4				39			39.2	39.4	39.1
	38.8		39.3	39.3	39.4				39			39.3	39.4	39
	39.3		39.6	39.3	38.5				39.2			39.7	39.6	39.2
	39.2		39.3	39.2	38.9				39.1			39.5	39.3	39
	38.8		39.3	39	38.4				39.3			39.6	39.2	39.1
	38.6		39.5	39.4	38.6				38.6			39.3	39.1	39
	38.9		39.1	39.5	38.6				38.9			39.6	39.4	39.2
	39.2		39.3	39.1	38.8				39.2			39.1	39.3	39.2
	38.5		39	39.4	39				39			39.2	39.3	39.1

Appendix 5.7

sh. 175	sh. 3	sh. 8	sh.13	sh. 86	sh. 101	sh. 104	sh. 105	sh. 111	sh. 132	sh. 137	sh. 141	sh. 153	sh. 160
38.8													
39													
39.1													
39.3													
38.7													
38.5													
38.6													
39													
40.1													
40.8													
										39.2		38.8	
	38.9	39.4	38.8		38.7	38.8	38.9	38.6	38.9	39.2	39.1	39	39.2
	39.1	39.8	39		38.8	39.2	39	38.8	39.3	39	38.8	39	39.1
	39.2	38.9	39.1	39.4	39	38.8	38.6	39.1	38.8	38.8	39.5	39	38.5
	39	39.8	39.3	39.7	39.4	39.5	39.1	38.8	39.5	39.5	39.3	39.4	39.1
	38.2	39.3	39	39.3	39.4	38.6	38.8	38.1	39	38.9	39	38.7	39.3
	38.5	39.2	39	39.2	39	38	38.6	38.4	39.2	38.5	38.7	38.9	39.2
	39	40.9	41	39.2	38.3	38.3	39.4	39.6	39.2	38.8	38.9	38.8	38.3
	41.5	39.9	40.6	39.8	41	40.7	41	40.6	39.2	39.5	39	39.2	39.3
	40.4	39.5	39.5	39.3	40.6	40.4	41	40.2	38.8	38.8	38.6	39	39.3
	39.6	39.4	39.4	39.4	40.8	41.1	41.1	39.3	38.8	39.3	39.3	39.2	39.3
	40.2	39.6	38.9	39.7	39.7	40.8	40.9	39.5	38.9	39.4	39.2	38.7	39.6
	39.7	39.9	39.4	41.3	39.8	40.2	40.1	39.6	39.2	39.5	39.5	39.1	39.4
	39.5	39.3	38.7	40.6	39.1	39.1	38.6	38.9	39.4	39.3	39.4	39.3	39.4
	38.9	39	38.6	40.4	39	39.5	38.3	38.6	39.1	39.4	40.5	39.2	41.7
	39.1	39.1	38.6	40.1	38.7	39.5	39.5	39	40.6	39.4	39.4	39.5	41
	38.8	38.6	39	40.1	39	39.5	39.5	39.3	40.7	39.2	39.8	38.7	40.4
	39.3	39.5	38.6	39.3	39.3	39.1	39.2	38.9	40.6	39.5	39.5	38.7	40.2
	39.3	39	38.5	39.2	38.6	39.4	39.3	39.5	38.9	39.4	39.3	39.2	39.3
	39.6	38.9	39	39.5	38.8	39.6	39.9	39.1	39	39.2	39	39	39.5
	39.2	38.7	39	39.2	38.9	39	39.1	38.9	38.8	39	38.9	39.1	39.3
	38.6	39.1	38.9	39	38.6	39.1	38.8	38.5	38.7	39	39	38.7	38.8
	38.6	38.8	39	39.5	38.9	39.2	39	38.9	38.5	39.1	39.5	39.5	39.2
	38.5	39.3	39	39.3	38.8	39.7	39.1	38.7	39	39.2	39	39.3	39.6
	39.1	39.1	39.1	39.3	39.1	38.9	39	39	38.9	39	39	39.3	39.2
									39.1	38.8	38.6	38.9	39.1
	38.7	38.6	38.8	39	38.7	39.1	38.8	38.9	39	39	38	39.1	39.2
	38.9	38.6	39.2	38.9	38.9	38.8	39	39	39.3	38.7	38.4	39.2	39.1
	38.5	39.3	38.5	38.8	39.1	39.5	38.7	39.4	38.9	39	38.4	38.8	39.4
	38.7	39.2	38.7	39	39	39.5	38.8	39.4	39	38.7	38.6	39.1	39.5
	38.8	39.2	38.9	39	39.1	39.4	38.9	39.3	39.1	38.7	38.6	39	39.5
	39	*39.1*	*39.1*	*39.2*	*39.2*	*39.4*	*39*	*39.3*	*39.3*	*38.6*	*38.8*	*39.3*	*39.3*
	40.2	38.9	38.6	39.7	38.8	39	39.1	39.1	38.9	39.2	39	38.7	39.2
	40.8	38.7	39	39.3	38.5	38.8	39.3	39	38.7	39.2	38.6	39.1	39.2
	40.4	38.8	39.1	39.7	39.3	39.2	38.9	39.2	39.2	39.3	38.7	39	39.2
	38.7	39	39.2	39.4	39.9	39.5	38.4	38.8	38.6	39.2	38.6	39	38.8
	38.9	38.8	39.2	39.2	38.5	39.2	39	38.6	38.8	39	38.9	39	39.3
	39	39	38.8	39	40	39.3	38.8	39	38.9	39.1	38.8	39	39.2
	39.1	38.9	39	39.7	39.1	39.3	39	38.9	39	39.2	38.7	39.4	39.4
	40.5	39.2	39	39.2	38.8	39.8	39.2	39.2	39.2	39.3	39	39.6	39.3
	39	39	39.2	39.6	38.9	39.6	39.2	38.8	38.9	39.5	39.5	38.9	39.5
	39.2	39.1	39.5	39.4	38.7	39.6	39.3	39	38.7	39	39.5	38.9	39.5
	38.9	38.7	39.1	39	38.5	39.4	39.2	38.8	38.7	39.1	39.1	38.4	39.4
	38.4	38.9	39.9	39.4	38.5	40.1	39.7	38.9	39.4	39.4	38.8	39.6	40.1
	39	39.3	38.5	40.1	38.8	40.6	40	38.5	38.8	38.8	39	39.4	39.3
	39.3	39.1	38.5	39.3	38.8	39.5	39.8	39.2	38.8	39	39	38.9	39.2
	39.5	39	39.4	39.5	39	39.6	39.6	39	39	39	39.1	38.8	39.6
	38.7	38.9	39.7	39.4	38.7	39.3	40.5	39	39	38.9	39	39.3	38.8
	38.7	39.1	39	39	38.9	39.3	39	38.7	38.6	38.9	38.8	39.4	39.5
	38.6	39.2	40	39.4	39	39.3	39.3	39.4	38.6	39	38.6	38.9	39.4
	38.6	39	39.7	39	38.6	39.5	39.4	39	39	39.2	38.9	39	39.2
	38.5	39	39.5	38.7	38.7	39.4	39.4	39	39.1	39.1	38.7	39	39.2
	38.8	39	39.3	38.9	38.9	39.5	39.6	39	39	39	38.9	38.8	39.3
	39.2	*39.4*	*39.6*	*39.3*	*39.2*	*39.3*	*39.3*	*39.2*	*38.8*	*39.2*	*39.2*	*39*	*39.1*
	39	38.8	38.8	39.7	38.8	39.9	39.2	39.2	39.2	39	38.7	39.6	39
	38.6	39.2	39.3	39.7	38.9	39.3	39.3	39.1	38.9	39.4	39	39.1	39.3
	38.6	39.3	39.1	39.4	39.1	39.8	39	39.2	39.5	39.2	38.8	39.1	39.5
	38.6	39.3	39.2	39.4	38.9	39.6	38.7	39.1	39.3	39.2	39	39.1	39.7
	39	39.1	38.7	39.5	39.3	39.7	39.4	39.4	39.2	39.1	39.1	39.1	38.9
	39	39.1	38.9	39.4	39	39.4	39.3	39.6	39	39	39	39.1	39.1
	38.5	38.6	38.5	39.1	39	39.2	38.2	38.8	39.1	38.7	39	39.2	39.4
	38.9	39.2	38.8	39.5	39	39.7	39.4	39	39.2	39.3	38.7	39.2	39.5
	38.8	39.3	39.1	39.3	39.2	39.8	39.6	39.1	39.2	39.3	39.1	39.5	39.5
	38.7	39.3	38.8	39	38.9	39.4	38.7	39.3	38.9	39.2	39	39.5	39.4
	39.9	39	38.4	39.4	38.8	39	39.2	39.4	39.1	39	39	39.6	39.1
	41	39	39.3	39.6	38.8	39.4	39.2	39.6	39.1	38.8	38.9	40.6	39.1
	41.8	39	38.7	39.2	38.9	39.5	40	39.9	39.1	39.3	38.9	41.7	39.6
	40.8	39.1	39.1	39.8	39	40.1	40.6	41.5	39.3	39.5	39.1	40.5	40.6
	41.3	39	39	39.6	41.1	41.3	40.9	41	39.3	40.2	38.9	41.2	41.2
	41.3	40.7	38.7	39.4	41.8	39.7	40.7	41.7	39	39.3	38.9	41.2	41.3
	40.6	39.3	38.5	39.7	41.6	39.8	40.4	41.3	39.2	39	38.8	40.7	41.5
	40	38.9	39	39.5	41	39.2	39.5	40.5	39.5	39.3	39	40.3	40.7
	40.2	3.3	38.9	39.7	40.8	39.2	39.4	39.6	39.7	39.2	39.3	39.5	40.6
	39.3	39.1	39.1	39.9	39.2	39.7	39.3	39.2	39.6	39.7	39.2	39.3	40.1
	40.3	39.2	39	39.2	38.9	39.4	38.5	39.5	39.3	38.8	39	39	39.6
	40.3	39.4	39	40.1	38.7	38.8	39	39.5	39.3	39.6	39.2	39.2	39.7
	39.7	39	38.9	39.3	39.4	39.5	39.2	39.6	39.5	39.6	39.3	39.2	39.1
	39.9	38.6	39.1	39.5	38.4	39.3	38.7	39.1	39.2	38.5	38.7	38.8	39.3
	39.4	39	39.4	39.3	38.6	38.8	38.5	39.2	39.3	39	39.1	39	39.3
	39.6	39.3	39.5	39.4	39	38.6	38.9	39.1	39.3	38.8	38.7	39.3	38.9
	39.5	39.	39	39.3	38.7	39.6	39	39.1	39	39.3	38.9	39.2	39
	39.4	39	39.3	39.2	38.6	39.4	39.2	39.3	39.4	39.1	39	38.8	38.8

Appendix 5.8 One way cross immunity trial: Temperatures of sheep during primary infections and challenge infections

Primary infection	Imm/Chal	Ase/Bar	Ase/Isio	Ase/Kis	Ase/Sus	Bam/Galg233	Bam/Isio	Bam/Kath32
	Day of infection	**sh 20**	**sh 62**	**sh 177**	**sh 63**	**sh 55**	**sh 57**	**sh 183**
	0	38.9	39.8	39.3	39.7	39.5	39.3	39.6
	1	39.6	39.9	39	39.5	39.5	39.3	39.4
	2	39.5	39.2	38.8	39.2	39.4	39.2	39.4
	3	39	39.3	39.1	39	39.8	39.2	39.5
	4	39.4	39.8	39.7	39.2	38.8	39.8	39.6
	5	39.2	39.7	39.5	40.2	39.2	39.1	39.4
	6	38.8	40	39.6	39.8	39.3	39.4	39.5
	7	38.6	40.2	39.3	39.6	39	39.1	38.6
	8	39.2	39.7	38.8	39.5	39	39.2	38.8
	9	39.5	39.5	38.8	40.2	38.9	38.6	39.3
	10	39.5	39	38.7	40.2	38.6	39	39.2
	11	40.5	39.3	38.7	40.6	39.2	38.9	39.2
	12	41.6	40.6	38.8	41.8	38.8	39	39.1
	13	41.8	41.8	38.9	41.6	39.6	39.4	39.4
	14	41.5	41.6	39.3	41.8	39.2	38.5	39.2
	15	41.6	41.6	39.4	41.1	40.2	39.7	38.6
	16	41.7	41	41	40.3	41.5	40	38.7
	17	40.2	40.6	41.4	39.9	41.9	41.6	39.1
	18	40.1	40.2	41.9	40.2	41	41.4	41.3
	19	40.5	38.8	41.7	38.7	40.9	40.3	40.8
	20	40	40.1	41	39.7	40.5	38.9	39.6
	21	40.2	39.2	40.2	39.3	40.5	39.2	41.3
	22	40.3	39	40	39.4	39.7	38.7	41.3
	23	40	39.5	39.8	39.4	39.3	39.4	40.6
	24	40	39.3	40.2	39.1	39.1	39.5	40.5
	25	40.2	38.7	39.5	39.4	38.9	38.6	40.6
	26	39.2	38.5	39.6	39.3	39.2	39.3	39.5
	27	39.6	38.4	39	-	39.4	39	39.4
Challenge infection								
	0	39.4	38.6	39	39	38.7	39.8	39.2
	1	39.5	38.6	38.7	38.7	38.6	39.2	38.6
	2	39	39.5	39.3	38.9	39.2	39.1	38.9
	3	39.2	39.6	39.1	39.2	39.5	39.8	39.2
	4	38.6	38.4	39.1	38.4	38.9	39.5	39.1
	5	38.7	38.7	39.1	38	39.2	39.3	39.3
	6	39.7	38.7	39.3	38	39.4	39.3	39.5
	7	39.6	38.9	39.1	38.5	38.9	39	38.8
	8	39.9	39.1	39.4	38.7	38.9	39.3	38.7
	9	39.2	39.3	39	38.8	39	39.2	39
	10	40.4	38.5	39.7	38.8	38.8	39	39.4
	11	41.6	38.7	39.5	38.6	39	39.5	39.1
	12	42	38.5	39.5	40.6	39.6	40	39.3
	13	40.4	41.2	40.8	40.8	39.3	41	39.1
	14	*	41	41.6	41.5	40.2	40.6	39.1
	15		41	41.8	40.2	41.2	40.2	39.3
	16		41.1	40.9	*	41.3	40.5	39.4
	17		41.6	*		41.3	40.9	39.3
	18		40.7			40	40	39.1
	19		39.7			40.8	39.8	39.2
	20		39.8			40.8	39.7	39.3
	21		39.9			40	39.2	39
	22		39.7			39.9	39.4	39.6
	23		39.8			39.2	39.5	39.3
	24		40.4			38.8	39	39
	25		39			38.9	39.3	39.9
	26		39.3			38.7	38.8	39.2
	27		39.5			38.9	39.1	39.2
	28		39.3			39	39.2	-

Bam/Kath32	Bam/Kath972	Bam/Mar	Bar/Sus	Gal233/Kath972	Isio/Gal233	Kath32/Kath972	Kath32/Sus
sh 187	**sh 34**	**sh 83**	**sh 59**	**sh 61**	**sh 38**	**sh 58**	**sh 35**
39.7	-	39.3	39	39.1	na	na	na
39.6	39.3	39.3	38.8	39.1	39.2	40	39.1
39.3	39.2	39.5	39.3	38.9	39	39	39.6
39.5	38.9	39.7	38.7	39	39	39.2	39.5
39	38.4	38.9	39	39.1	39.3	38.5	39.4
39.3	39.3	39	39.2	39.2	39	39	39.4
39.4	39	39	38.9	39.2	38.8	39.5	39.2
39	39.5	38.3	39.1	39.3	39.2	39.5	39
39.2	39.9	39	40.6	39.4	39	39.5	39
39.1	40.1	39.3	41.2	39.2	39.2	39	39.3
39.2	40.2	39.1	41.1	39.1	39.6	40.1	39.2
39.1	39	39	40.6	38.9	39.3	39.9	39.5
38.9	39.3	39.2	40	39.1	39.6	39	39.5
39	39	38.6	39.4	39.4	41.2	41.5	40.5
40.6	39.9	38.8	39.1	39.5	41.6	41.6	40.6
41.1	41.2	39.2	39.4	39.6	41.7	41	41.4
41.4	41.4	39.3	38.9	41.6	41.1	41.7	41.6
41.7	40.2	41.2	38.9	41.4	41	39.9	41.6
41	41.6	41.3	39	41.7	40.8	39.7	41.2
40.6	41.2	40.6	39.1	40.6	39.2	39.9	39.6
39.5	40.9	41	39.3	39.6	38.4	39.3	38.9
39.7	40.9	41.9	39	39.6	39.2	40	39.2
41	40.6	41.3	38.1	39.7	39.3	38.9	39.5
40.4	40.1	40.3	38.5	39	38.8	39.9	39.3
40.2	39.9	40.9	39	38.9	39	40	39.2
40.9	38.5	40.5	39	38.8	39.3	39.5	39.5
39.5	39.8	40.2	39.2	-	39	39.3	39.3
39.3	39.3	40.3	39.2	-	38.9	39.6	38.7
39.2	38.9	39.3	39.4	38.9	38.8	39.4	39.2
38.7	39.2	39.2	38.4	38.7	39.5	38.9	39.1
39	39.1	38.7	39	38.9	39.3	38.9	39.3
38.9	39.2	38.8	39.7	39.3	39.7	39	38.7
39.2	39.2	39.6	39.7	38.9	39.4	38.2	39.6
39.3	38.7	39	39.4	39	39.2	38.6	39.2
39.4	39.2	39.7	39.2	39.1	39	38.8	39.4
39	39.3	39.2	39.2	39	39	38.8	39.3
39.3	39.1	39.1	39	38.8	39	39.2	39.8
39.5	39.5	38.9	38.9	38.8	39	39	40.6
39	39.1	39	39	38	39	38.9	41.4
39.1	39.2	39.5	39.5	38.8	39.1	40.6 *	
39.7	39.8	38.8	39.5	39	39.4	40.6	
39.7	41.8	39.5	38.7	38.9	39.6	41.5	
39.3	40.8	39.7	39.2	39.5	39.4	41.6	
39.2	41.4	39.5	39.4	39.4	39.2	41.1	
39.3	41.6	39.5	38.9	38.7	39.8	41.1	
39.1	41.3	39.2	39.2	39	40 *		
38.8	40.2	40	38.9	38.8	39.7		
39.8	40.3	39.5	39	39.2	39.3		
39.6	39.4	40	39	39	39.8		
39.4	38.9	40	39.2	40.8	39.5		
39.3	39.2	39.9	39.1	39.6	39.2		
39.1	39.4	39.4	38.9	39.5	39.1		
38.9	39	39	39.2	40.5	39		
39.7	39.5	38.7	39.1	39	39.2		
39.6	39.2	38.6	39.2	39.8	39		
39.1	39.5	38.9	39.2	39.6	39		
-	39.2	-	39	39.5	39.1		

Kath972/Bar	Kis/Ase	Mar/Bam	Mar/Bar	Sus/Kis
sh 46	**sh 33**	**sh 47**	**sh 48**	**sh 54**
39.2	38.5	37.9	39.3	na
39	39.7	38.4	39.7	39.6
39	39.7	38.5	39.4	39.6
39.2	39.4	38	39.5	39.1
39.3	39	38.7	39.3	39.4
39.4	39.6	38.7	39.8	39.3
39.2	39.9	38.1	39.6	39
39.2	40.3	39	39.3	39
39.4	40.8	39.1	39.7	39.5
39	41.3	39.2	39.7	40.7
41.3	41.5	38.5	39.7	40.6
41	41.1	39	39.6	41.6
41.2	40.1	38.6	40.7	39.1
41.6	39.9	38.5	41.1	39.9
40.9	39.5	40.4	41.3	38.8
40.3	39.4	40.9	41.6	39.4
39.3	39.4	40.5	41.2	39
39.2	39	41.4	40.3	38.7
39	38.8	42	39.6	39.2
39.5	39.5	41.3	39.4	39.2
39	38.6	39.8	39	39.6
39.5	39.6	39.2	39.7	38.8
39.5	38.7	39.8	39.3	39
38.9	38.6	39.4	39.2	39.2
39.2	38.6	39.7	39.3	39.8
39.3	38.8	38.5	39.2	39.6
38.6	38.9	39.2	39.5	39.5
38.6	38.5	38.4	39.2	39.4
38.5	39.4	38.9	38.8	39.1
39.5	39.3	38.7	38.6	38.6
39.3	38.9	39.2	39.2	39.5
39.1	38.9	39.3	39.2	39.8
38.9	38.7	38.5	38.3	39.9
38.9	39.1	38.8	38.5	39.5
39	39	38.8	38.5	39.6
39	39.3	38.6	38.7	39.7
38.8	38.9	39	38.9	39.8
38.8	39.2	38.2	39	39.5
38	39.2	38.7	39.2	39.2
38.8	39.3	39.2	41	39.2
38.8	39.9	39.3	39.1	39.5
38.9	41.5	39.1	39.6	39.4
39.4	42	38.9	39.1	39
39.3	41.8	39.1	39.2	39.2
38.8	41.2	39.1	38.8	39.5
39.2	40.6	39.2	38.9	39.5
39.5	40.4	39.1	39	39.5
39.2	39.5	39.3	39.8	39.6
39.3	39.5	39	39	39.4
39.2	39.6	38.5	39.2	39.7
39.2	39.2	38.8	39.3	39.3
39	40	39	39.2	39
38.8	40	38.7	39.5	39.2
38.7	39.7	38.7	39.4	39.1
39	39.5	38.9	39.5	38.9
38.9	39.4	38.8	39.7	39
38.6 -	-		39.5 -	

Appendix 6.1 The serological response (OD values) of mice innoculated with fresh infective sheep blood.

(a)

suswa /mouse	pre-infection	post-infection	sero-conversion
c2bm2	0.136	0.109	-
c2bm6	0.081	0.104	-
c3bm3	0.17	0.23	+
c4bm1	0.085	0.347	+
c1bm1	0.069	0.171	+
c1bm2	0.108	0.319	+
c1bm3	0.258	0.09	-
c1bm4	0.137	0.14	-
c1bm5	0.137	0.232	+
c2bm1	0.163	0.363	+
c2bm3	0.172	0.264	+
c2bm4	0.002	0.129	-*
c2bm5	0.096	0.158	+
c3bm1	0.119	0.196	+
c3bm2	0.095	0.15	+
c3bm4	0.13	0.221	+
c3bm5	0.163	0.303	+
c3bm6	0.07	0.286	+
c4bm2	0.099	0.251	+
c4bm3	0.108	0.28	+
c4bm4	0.061	0.085	-
c4bm5	0.106	0.237	+
c4bm6	0.077	0.216	+

(b)

Baragoi/ mouse	pre-infection	post-infection	sero-conversion
c4cm2	0.009	0.043	-
c4cm3	0.004	✞	
c4cm6	0.088	✞	
c5cm3	0.046	✞	
c5cm4	0.081	✞	
c5cm6	0.096	0.346	+
c4cm4	0.021	0.139	-
c5cm1	0.152	✞	
c5cm2	0.006	0.236	+
c5cm5	0.064	0.118	-

KEY

cut off OD = 0.143; +ve OD control = 0.75;

* value was at least x four the pre-infection value

✞ -death; nd - not determined; ns no serum

(c)

Kath972/ mouse	pre-infection	post-infection	sero-conversion
c4m1	0.018	0.054	-
c4m2	0.295	0.131	-
c4m4	0.031	✞	
c4m6	nd	0.045	-
c5m6	0.07	0.079	-
c4m3	ns	0.085	-
c4m5	nd	0.057	-
c5m1	0.076	0.041	-
c5m2	0.112	0.03	-
c5m3	0.077	0.196	+
c5m4	0.076	0.024	-
c5m5	0.049	0.066	-

(d)

Kiswani/ mouse	pre-infection	post-infection	sero-conversion
c3xm1	0.07	0.053	-
c3xm3	0.078	0.254	+
c3xm4	0.138	0.171	+
c4xm1	0.029	0.016	-
c4xm2	0.065	0.061	-
c4xm4	0.108	0.08	-
c3xm2	0.036	0.01	-
c3xm5	0.045	0.03	-

(e)

Gal229/ mouse	pre-infection	post-infection	sero-conversion
c4xm3	0.061	0.098	-
c5xm1	0.092	0.713	+
c5xm2	0.051	0.817	+
c5xm4	0.052	0.056	-
c6bm4	0.103	0.047	-
c7bm3	0.159	0.064	-
c6bm1	0.114	0.069	-
c6bm2	0.031	0.061	-
c6bm3	0.046	nd	
c4xm5	0.077	0.091	-
c5xm3	0.045	0.086	-
c5xm5	ns	0.062	-
c6bm5	0.023	0.087	-
c7bm1	0.089	0.101	-
c7bm2	0.105	0.073	-
c7bm4	0.083	0.079	-
c7bm5	0.008	0.088	-
c7bm6	0.036	0.057	-

(f)

Gal233/ mouse	pre-infection	post-infection	sero-conversion
c3dm1	0.073	0.291	+
c4dm2	0.134	0.308	+
c4dm3	0.206	0.400	+
c4dm6	0.16	0.316	+
c5dm1	0.117	0.321	+
c3dm2	0.095	0.163	+
c3dm3	0.168	0.213	+
c3dm4	0.146	0.130	-
c3dm5	0.299	0.183	+
c3dm6	0.129	0.183	+
c4dm1	0.244	0.263	+
c4dm4	0.236	0.183	+
c4dm5	0.198	0.165	+
c5dm4	0.088	0.071	-

(g)

Isiolo/ mouse	pre-infection	post-infection	sero-conversion
c1m1	ns	0.444	+
c1m2	ns	0.291	+
c2m1	0.031	0.387	+
c2m2	0.114	0.557	+
c3m1	0.091	0.526	+
c3m3	0.017	0.316	+
c1m3	0.057	0.263	+
c1m4	0.024	0.077	-
c1m5	0.137	0.170	+
c2m3	0.133	0.078	+
c2m5	ns	0.171	+
c3m4	0.106	0.126	-
c3m5	0.228	0.412	+

(h)

Kath- 32 /mouse	pre-infection	post-infection	sero-conversion
c3cm1	nd	0.214	+
c3cm2	nd	0.284	+
c3cm3	nd	0.178	+
c3cm4	0.067	0.138	-
c4cm1	0.061	0.185	+
c3cm5	0.067	0.166	+
c3cm6	0.087	0.194	+
c4cm5	0.014	0.199	+

(i)

Asembo/ mouse	pre-infection	post-infection	sero-conversion
c1dm1	0.12	0.145	+
c1dm2	0.035	↟	
c2dm1	0.059	↟	
c2dm4	0.041	↟	
c2dm5	0.222	0.425	+
c2dm6	0.125	↟	
c1dm3	0.202	0.137	-
c1dm4	0.06	↟	
c1dm5	0.083	↟	
c1dm6	0.113	0.274	+
c2dm2	0.143	0.06	-
c2dm3	0.156	0.07	-

(j)

Marigat/ mouse	pre-infection	post-infection	sero-conversion
c1xm3	0.075	0.072	-
c1xm4	0.126	0.053	-
c1xm5	0.028	0.051	-
c2xm2	0.068	0.116	-
c2xm5	0.06	0.117	-
c1xm1	0.045	0.061	-
c1xm2	0.046	0.016	-
c2xm1	0.095	0.039	-
c2xm3	0.055	0.063	-
c2xm4	0.083	0.086	-

(k)

Bamba/ mouse	pre-infection	post-infection	sero-conversion
c1cm1	0.087	0.226	+
c1cm4	0.043	0.166	+
c2cm2	0.037	0.15	+
c2cm3	0.031	0.146	+
c2cm4	0.069	0.122	-
c2cm6	nd	0.172	+
c1cm2	0.025	0.134	-
c1cm3	0.022	nd	
c1cm5	0.086	0.112	-
c1cm6	0.028	0.058	-
c2cm1	0.043	0.191	+
c2cm5	0.033	0.095	-

Appendix 7.1 *Amblyomma variegatum* and *A gemma* tick drops from sheep infected with *Cowdria ruminantium* (Asembo and Bamba) isolates and the tick selection for dissection.

Sheep number	Date applied	Date collected	Number dropped	Ticks dissected	Number dropped	Ticks dissected	Sheep status
91	30.11.95		LS Av		LS Ag		sero +ve
av	2.12.95	5-Dec	0	0	32	10	
(Asembo)		6-Dec	67	10	109	10	
		7-Dec	58	10	66	10	
		8-Dec	47	10	56	10	
		9-Dec	33	10	28	10	
total			205	40	291	50	
			LS Av		LS Ag		
102	13.12.95	19-Dec	0	-	14	0	sero -ve
ag	15.12.95	20-Dec	220	0	26	0	
	17.12.95	21-Dec	230	0	34	0	
		22-Dec	96	10	39	10	
		23-Dec	7	0	12	0	
		24-Dec	0	0	13	0	
		25-Dec	0	0	0	0	
		26-Dec	48	0	0	0	
		27-Dec	103	10	32	10	
		28-Dec	24	0	34	0	
		29-Dec	11	0	23	0	
total			739	20	227	20	
			ls Av 70		LS Ag 4		
177	3.4.96	8-Apr	0	0	8	8	sero +ve
(Asembo)		9-Apr	29	10	174	10	
		10-Apr	108	10	123	10	
		11-Apr	57	10	24	10	
		12-Apr	6	6	16	10	
		13-Apr	0	0	2	2	
total			200	36	347	50	
			LS Av 70		LS Ag 4		
177	4.3.96	9-Apr	0	0	23	10	
(Asembo)		10-Apr	8	8	170	10	
		11-Apr	30	10	110	10	
		12-Apr	10	10	24	10	
		13-Apr	2	2	5	5	
total			50	30	332	45	
			ls Av 69		LS Ag3		
176	3.4.96	8-Apr	0	0	3	3	sero +ve
(Asembo)		9-Apr	14	10	140	10	
		10-Apr	113	10	140	10	
		11-Apr	27	10	23	10	
		12-Apr	6	6	11	10	
total			160	36	317	43	
		9-Apr	0	0	15	10	
176	4.4.96	10-Apr	14	10	139	10	
(Asembo)		11-Apr	20	10	100	10	
		12-Apr	15	10	43	10	
		13-Apr	4	4	6	6	
		14-Apr	0	0	3	3	
		15-Apr	2	2	0	0	
		16-Apr	0	0	0	0	
		17-Apr	0	0	5	5	
total			55	36	311	54	
			LS Av 71		LS Ag 5		
183	7.4.96	12-Apr	0	0	3	3	sero +ve
(Bamba)		13-Apr	7	7	16	10	
		14-Apr	27	10	50	10	
		15-Apr	2	2	24	10	
		16-Apr	0	0	15	10	
		17-Apr	0	0	4	4	
		18-Apr	0	0	4	4	
tc tal			36	19	116	51	
			LS Av 71		LS Ag 5		
		15-Apr	0	0	167	10	
183	9.4.96	16-Apr	7	7	328	10	
(Bamba)		17-Apr	8	8	51	10	
		18-Apr	2	2	24	10	
		19-Apr	1	1	12	10	
total			18	18	582	50	
			LS Av 72		LS Ag 6		
187	7.4.96	12-Apr	0	nd	4	nd	sero +ve
(Bamba)		13-Apr	0	nd	19	nd	

Sheep number	Date applied	Date collected	Number dropped	Ticks dissected	Number dropped	Ticks dissected	Sheep status
		14-Apr	0	nd	17	nd	
		15-Apr	2	nd	2	nd	
		16-Apr	0	nd	2	nd	
		17-Apr	0	nd	1	nd	
		18-Apr	0	nd	1	nd	
total			2	0	46	0	
			LS Av 72		LS Ag 6		
187	9.4.96	15-Apr	0	nd	81	nd	
(Bamba)		16-Apr	1	nd	250	nd	
		17-Apr	0	nd	119	nd	
		18-Apr	0	nd	28	nd	
		19-Apr	0	nd	20	nd	
total			1	0	498	0	

KEY

Asembo - isolated from A variegatum; Bamba - isolated from A gemma

LS Av 71 = laboratory stock of *Amblyomma variegatum* lot number seventy one.

LS Ag 6 = laboratory stock of *Amblyomma gemma* lot number six

nd = not done.

Appendix 7.2: Concentration of DNA (absorbance at 260 λ) of ticks selected for comparing *Cowdria* infection rates

Sheep #	Date	Patch #	Species	Sample #	Absorbance with 5 µl sample	mg/ml	Absorbance with 10 µl sample	mg/ml
8	15/11/95	1	Av	97	0.222	1110	0.57	1425
8	15/11/95	1	Av	98	0.326	1630	0.615	1537.5
8	15/11/95	1	Av	99	0.365	1825	0.432	1080
8	15/11/95	1	Av	100	0.142	710	0.29	725
104	15/11/95	1	Av	91	0.292	1460	0.672	1680
104	15/11/95	1	Av	92	0.284	1420	0.546	1365
104	15/11/95	1	Av	93	0.252	1260	0.48	1200
104	15/11/95	1	Av	94	0.306	1530	0.58	1450
104	15/11/95	1	Av	95	0.307	1535	0.574	1435
104	15/11/95	1	Av	96	0.15	750	0.307	767.5
177	9/4/96	1	Av	101	0.28	1400	0.533	1332.5
177	9/4/96	1	Av	102	0.158	790	0.389	972.5
177	9/4/96	1	Av	103	0.231	1155	0.354	885
177	9/4/96	1	Av	104	0.276	1380	0.516	1290
177	9/4/96	1	Av	105	0.245	1225	0.438	1095
177	9/4/96	1	Av	106	0.289	1445	0.565	1412.5
177	9/4/96	1	Av	107	0.261	1305	0.469	1172.5
177	9/4/96	1	Av	108	0.237	1185	0.417	1042.5
177	9/4/96	1	Av	109	0.309	1545	0.492	1230
177	9/4/96	1	Av	110	0.304	1520	0.503	1257.5
177	10/4/96	1	Av	121	0.199	995	0.336	840
177	10/4/96	1	Av	122	0.232	1160	0.399	997.5
177	10/4/96	1	Av	123	0.256	1280	0.397	992.5
177	10/4/96	1	Av	124	0.289	1445	0.521	1302.5
177	10/4/96	1	Av	125	0.289	1445	0.531	1327.5
177	10/4/96	1	Av	126	0.252	1260	0.427	1067.5
177	10/4/96	1	Av	127	0.322	1610	0.554	1385
177	10/4/96	1	Av	128	0.298	1490	0.536	1340
177	10/4/96	1	Av	129	0.387	1935	0.615	1537.5
177	10/4/96	1	Av	130	0.267	1335	0.5	1250
177	11/4/96	1	Av	141	0.267	1335	0.441	1102.5
177	11/4/96	1	Av	142	0.289	1445	0.475	1187.5
177	11/4/96	1	Av	144	0.304	1520	0.528	1320
177	11/4/96	1	Av	145	0.239	1195	0.402	1005
177	11/4/96	1	Av	146	0.234	1170	0.422	1055
177	11/4/96	1	Av	147	0.351	1755	0.587	1467.5
177	11/4/96	1	Av	148	0.335	1675	0.605	1512.5
177	11/4/96	1	Av	149	0.323	1615	0.562	1405
177	11/4/96	1	Av	150	0.264	1320	0.472	1180
177	12/4/96	1	Av	159	0.285	1425	0.505	1262.5
177	12/4/96	1	Av	160	0.354	1770	0.44	1100
177	12/4/96	1	Av	161	0.272	1360	0.502	1255
177	12/4/96	1	Av	162	0.372	1860	0.688	1720
177	12/4/96	1	Av	163	0.295	1475	0.473	1182.5
177	12/4/96	1	Av	164	0.427	2135	0.612	1530
177	9/4/96	2	Ag	111	0.27	1350	0.46	1150
177	9/4/96	2	Ag	112	0.359	1795	0.674	1685
177	9/4/96	2	Ag	113	0.292	1460	0.483	1207.5
177	9/4/96	2	Ag	114	0.315	1575	0.47	1175
177	9/4/96	2	Ag	115	0.389	1945	0.615	1537.5
177	9/4/96	2	Ag	116	0.529	2645	0.834	2085
177	9/4/96	2	Ag	117	0.3	1500	0.524	1310
177	9/4/96	2	Ag	118	0.272	1360	0.428	1070
177	9/4/96	2	Ag	119	0.236	1180	0.503	1257.5
177	9/4/96	2	Ag	120	0.277	1385	0.434	1085
177	10/4/96	2	Ag	131	0.306	1530	0.506	1265
177	10/4/96	2	Ag	132	0.219	1095	0.401	1002.5
177	10/4/96	2	Ag	133	0.226	1130	0.426	1065
177	10/4/96	2	Ag	134	0.267	1335	0.481	1202.5
177	10/4/96	2	Ag	135	0.253	1265	0.515	1287.5
177	10/4/96	2	Ag	136	0.116	580	0.178	445
177	10/4/96	2	Ag	137	0.309	1545	0.488	1220
177	10/4/96	2	Ag	138	0.301	1505	0.507	1267.5
177	10/4/96	2	Ag	139	0.277	1385	0.48	1215
177	10/4/96	2	Ag	140	0.256	1280	0.442	1105
177	11/4/96	2	Ag	175	0.346	1730	0.533	1332.5
177	11/4/96	2	Ag	176	0.526	2630	0.441	1102.5
177	11/4/96	2	Ag	177	0.236	1180	0.431	1077.5
177	11/4/96	2	Ag	178	0.253	1265	0.396	990
177	11/4/96	2	Ag	179	0.286	1430	0.456	1140
177	11/4/96	2	Ag	180	0.215	1075	0.447	1117.5
177	11/4/96	2	Ag	181	0.218	1090	0.389	972.5
177	11/4/96	2	Ag	182	0.288	1440	0.447	1117.5
177	11/4/96	2	Ag	183	0.274	1370	0.471	1177.5
177	11/4/96	2	Ag	184	0.268	1340	0.444	1110

Sheep #	Date	Patch #	Species	Sample #	Absorbance with 5 µl sample	mg/ml	Absorbance with 10 µl sample	mg/ml
177	12/4/96	2	Ag	195	0.355	1775	0.575	1437.5
177	12/4/96	2	Ag	196	0.276	1380	0.47	1175
177	12/4/96	2	Ag	197	0.276	1380	0.462	1155
177	12/4/96	2	Ag	198	0.259	1295	0.451	1127.5
177	12/4/96	2	Ag	199	0.318	1590	0.501	1252.5
177	12/4/96	2	Ag	200	0.236	1180	0.402	1005
177	13/4/96	3	Av	214	0.242	1210	0.409	1022.5
177	13/4/96	3	Av	215	0.194	970	0.347	867.5
177	10/4/96	3	Av	205	0.245	1225	0.423	1057.5
177	10/4/96	3	Av	206	0.265	1325	0.446	1115
177	10/4/96	3	Av	207	0.266	1330	0.468	1170
177	10/4/96	3	Av	208	0.216	1080	0.39	975
177	10/4/96	3	Av	209	0.256	1280	0.404	1010
177	10/4/96	3	Av	210	0.306	1530	0.547	1367.5
177	10/4/96	3	Av	211	0.3	1500	0.507	1267.5
177	11/4/96	3	Av	165	0.314	1570	0.46	1150
177	11/4/96	3	Av	166	0.285	1425	0.481	1202.5
177	11/4/96	3	Av	167	0.283	1415	0.502	1255
177	11/4/96	3	Av	168	0.242	1210	0.421	1052.5
177	11/4/96	3	Av	169	0.3	1500	0.484	1210
177	11/4/96	3	Av	170	0.312	1560	0.515	1287.5
177	11/4/96	3	Av	171	0.286	1430	0.437	1092.5
177	11/4/96	3	Av	172	0.236	1180	0.392	980
177	11/4/96	3	Av	173	0.245	1225	0.414	1035
177	11/4/96	3	Av	174	0.286	1430	0.479	1197.5
177	12/4/96	3	Av	185	0.33	1650	0.543	1357.5
177	12/4/96	3	Av	186	0.265	1325	0.46	1150
177	12/4/96	3	Av	187	0.272	1360	0.481	1202.5
177	12/4/96	3	Av	188	0.264	1320	0.457	1142.5
177	12/4/96	3	Av	189	0.299	1495	0.533	1332.5
177	12/4/96	3	Av	190	0.254	1270	0.439	1097.5
177	12/4/96	3	Av	191	0.271	1355	0.473	1182.5
177	12/4/96	3	Av	192	0.362	1810	0.631	1577.5
177	12/4/96	3	Av	193	0.275	1375	0.479	1197.5
177	12/4/96	3	Av	194	0.389	1945	0.626	1565
177	13/4/96	4	Ag	256	0.259	1295	0.426	1065
177	13/4/96	4	Ag	260	0.241	1205	0.412	1030
177	10/4/96	4	Ag	226	0.201	1005	0.343	857.5
177	10/4/96	4	Ag	227	0.24	1200	0.418	1045
177	10/4/96	4	Ag	228	0.301	1505	0.457	1142.5
177	10/4/96	4	Ag	229	0.28	1400	0.489	1222.5
177	10/4/96	4	Ag	230	0.211	1055	0.387	967.5
177	10/4/96	4	Ag	231	0.332	1660	0.637	1592.5
177	10/4/96	4	Ag	232	0.212	1060	0.371	927.5
177	11/4/96	4	Ag	236	0.291	1455	0.501	1252.5
177	11/4/96	4	Ag	237	0.291	1455	0.476	1190
177	11/4/96	4	Ag	238	0.293	1465	0.473	1182.5
177	11/4/96	4	Ag	239	0.292	1460	0.51	1275
177	11/4/96	4	Ag	240	0.285	1425	0.482	1205
177	11/4/96	4	Ag	241	0.297	1485	0.524	1310
177	11/4/96	4	Ag	242	0.308	1540	0.573	1432.5
177	11/4/96	4	Ag	243	0.346	1730	0.543	1357.5
177	11/4/96	4	Ag	244	0.323	1615	0.547	1367.5
177	11/4/96	4	Ag	245	0.242	1210	0.414	1035
177	12/4/96	4	Ag	246	0.258	1290	0.44	1100
177	12/4/96	4	Ag	247	0.21	1050	0.439	1097.5
177	12/4/96	4	Ag	248	0.284	1420	0.501	1252.5
177	12/4/96	4	Ag	249	0.33	1650	0.559	1397.5
177	12/4/96	4	Ag	250	0.325	1625	0.53	1325
177	12/4/96	4	Ag	251	0.38	1900	0.614	1535
177	12/4/96	4	Ag	252	0.369	1845	0.574	1435
177	12/4/96	4	Ag	253	0.279	1395	0.461	1152.5
177	12/4/96	4	Ag	254	0.219	1095	0.48	1200
177	12/4/96	4	Ag	255	0.333	1665	0.502	1255
183	13/4/96	1	Av	261	0.257	1285	0.481	1202.5
183	13/4/96	1	Av	262	0.259	1295	0.483	1207.5
183	13/4/96	1	Av	263	0.248	1240	0.402	1005
183	13/4/96	1	Av	264	0.282	1410	0.4	1000
183	13/4/96	1	Av	265	0.332	1660	0.443	1107.5
183	13/4/96	1	Av	266	0.272	1360	0.539	1347.5
183	13/4/96	1	Av	267	0.267	1335	0.427	1067.5
183	14/4/96	1	Av	268	0.277	1385	0.421	1052.5
183	14/4/96	1	Av	269	0.254	1270	0.662	1655
183	14/4/96	1	Av	270	0.283	1415	0.604	1510
183	14/4/96	1	Av	271	0.404	2020	0.537	1342.5
183	14/4/96	1	Av	272	0.196	980	0.697	1742.5
183	14/4/96	1	Av	273	0.331	1655	0.61	1525
183	14/4/96	1	Av	274	0.238	1190	0.506	1265
183	14/4/96	1	Av	275	0.286	1430	0.471	1177.5

Sheep #	Date	Patch #	Species	Sample #	Absorbance with 5 µl sample	mg/ml	Absorbance with 10 µl sample	mg/ml
183	14/4/96	1	Av	276	0.264	1320	0.488	1220
183	14/4/96	1	Av	277	0.326	1630	0.602	1505
183	15/4/96	1	Av	278	0.294	1470	0.551	1377.5
183	15/4/96	1	Av	279	0.297	1485	0.559	1397.5
183	13/4/96	2	Ag	283	0.405	2025	0.504	1260
183	13/4/96	2	Ag	284	0.31	1550	0.559	1397.5
183	13/4/96	2	Ag	285	0.303	1515	0.44	1100
183	13/4/96	2	Ag	286	0.413	2065	0.812	2030
183	13/4/96	2	Ag	287	0.284	1420	0.544	1360
183	13/4/96	2	Ag	288	0.313	1565	0.572	1430
183	13/4/96	2	Ag	289	0.425	2125	0.761	1902.5
183	14/4/96	2	Ag	293	0.218	1090	0.571	1427.5
183	14/4/96	2	Ag	294	0.252	1260	0.417	1042.5
183	14/4/96	2	Ag	295	0.29	1450	0.539	1347.5
183	14/4/96	2	Ag	296	0.297	1485	0.511	1277.5
183	14/4/96	2	Ag	297	0.385	1925	0.672	1680
183	14/4/96	2	Ag	298	0.328	1640	0.628	1570
183	14/4/96	2	Ag	299	0.334	1670	0.529	1322.5
183	14/4/96	2	Ag	300	0.315	1575	0.581	1452.5
183	14/4/96	2	Ag	301	0.393	1965	0.574	1435
183	14/4/96	2	Ag	302	0.257	1285	0.421	1052.5
183	15/4/96	2	Ag	311	0.286	1430	0.47	1175
183	15/4/96	2	Ag	312	0.312	1560	0.543	1357.5
183	16/4/96	3	Av	330	0.179	895	0.344	860
183	16/4/96	3	Av	331	0.151	755	0.32	800
183	16/4/96	3	Av	332	0.268	1340	0.499	1247.5
183	16/4/96	3	Av	333	0.25	1250	0.479	1197.5
183	16/4/96	3	Av	334	0.161	805	0.346	865
183	16/4/96	3	Av	335	0.249	1245	0.519	1297.5
183	16/4/96	3	Av	336	0.266	1330	0.65	1625
183	17/4/96	3	Av	337	0.192	960	0.372	930
183	17/4/96	3	Av	338	0.189	945	0.332	830
183	17/4/96	3	Av	339	0.33	1650	0.548	1370
183	17/4/96	3	Av	340	0.267	1335	0.433	1082.5
183	17/4/96	3	Av	341	0.252	1260	0.445	1112.5
183	17/4/96	3	Av	342	0.289	1445	0.525	1312.5
183	17/4/96	3	Av	343	0.314	1570	0.517	1292.5
183	17/4/96	3	Av	344	0.326	1630	0.566	1415
183	18/4/96	3	Av	345	0.302	1510	0.568	1420
183	19/4/96	3	Av	346	0.268	1340	0.503	1257.5
183	16/4/96	4	Ag	357	0.26	1300	0.428	1070
183	16/4/96	4	Ag	358	0.251	1255	0.466	1165
183	16/4/96	4	Ag	359	0.261	1305	0.448	1120
183	16/4/96	4	Ag	362	0.233	1165	0.434	1085
183	16/4/96	4	Ag	363	0.268	1340	0.499	1247.5
183	17/4/96	4	Ag	367	0.258	1290	0.499	1247.5
183	17/4/96	4	Ag	368	0.276	1380	0.483	1207.5
183	17/4/96	4	Ag	369	0.226	1130	0.463	1157.5
183	17/4/96	4	Ag	372	0.221	1105	0.508	1270
183	17/4/96	4	Ag	373	0.243	1215	0.473	1182.5
183	17/4/96	4	Ag	374	0.243	1215	0.435	1087.5
183	18/4/96	4	Ag	382	0.382	1910	0.425	1062.5
183	19/4/96	4	Ag	392	0.236	1180	0.406	1015

Appendix 7.3 KB Ladder

Isolation of the causative agent of heartwater (*Cowdria ruminantium*) from three *Amblyomma* species in eight Districts of Kenya

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Thirteen isolates of *Cowdria ruminantium* were made from eight different Districts of Kenya by four different isolation methods. Feeding adult *Amblyomma* species ticks derived from nymphs collected in the field and the inoculation of homogenates prepared from adult field ticks had the highest success rate. The reattachment of adult ticks collected in the field was successful on only one of five attempts, and the subinoculation of blood from suspected heartwater carriers was unsuccessful. Seven of the isolates were derived from *A variegatum* ticks, four from *A gemma*, one from *A lepidum* and one from a mixed pool of the last two species. This is the first report of the isolation of *C ruminantium* from *A gemma* ticks, and the first report of its transtadial transmission from nymphal to adult *A gemma*.

HEARTWATER is an important disease of cattle, sheep, goats and wild ruminants (Uilenberg 1983) which is caused by a rickettsia, *Cowdria ruminantium* (Cowdry 1926a). After theileriosis it is the most important tick-borne disease as a major constraint to livestock production in Africa (Norval and others 1991). It is likely to become even more important in regions where immunisation against East Coast fever (*Theilera parva* infection) is being implemented and tick control is being reduced (Mutugi 1986). *C ruminantium* is transmitted by ticks of the *Amblyomma* genus (Cowdry 1926b) and is considered to be present in most of sub-Saharan Africa (Provost and Bezuidenhout 1987), where the vectors are distributed (Walker and Olwage 1987).

Infection with *C ruminantium* may result in a range of clinical reactions from peracute to acute, to mild or inapparent (Mare 1984). In the acute form, infected animals have a high fever and nervous signs, and there is high mortality. In the mild form, a fever is usually the only sign (Alexander 1931) and it may go unnoticed.

Although the causative organism and vector of heartwater have long been recognised (Cowdry 1926a, b), problems were encountered in the isolation and demonstration of the organism (Ilemobade and others 1975, Ilemobade and Blotkamp 1978) until tissue culture techniques (Bezuidenhout and others 1985, Logan and others 1987) and DNA probes (Waghela and others 1991, Mahan and others 1992) for *C ruminantium* were developed.

Susceptible animals are still required for the isolation of *C ruminantium* from the field and workers have used a variety of sources of infective material. Subinoculation of blood from clinical cases has been a reliable method (Du Plessis and Kumm 1971, Ilemobade 1976, Jongejan and others 1984) but the subinoculation of blood from recovered animals has been less satisfactory (Uilenberg 1971, Du Plessis and Bezuidenhout 1979). *Amblyomma* species ticks are the alternative source for the isolation of *C ruminantium* from the field. Adult ticks applied to susceptible hosts (MacKenzie and van Rooyen 1981) or inoculated as homogenates (Alexander 1931, Barre and others 1984) have been used successfully. However, owing to the apparently wide range of infection rates in the ticks, and the possibility of anaphylactic reactions to the inoculation of ground-up tick homogenates, the field isolation of *C ruminantium* is unreliable and requires perseverance, particularly where the clinical disease is not evident (FAO 1984).

Information on heartwater in Kenya is scanty (Daubney 1929-30, Morzaria and others 1983) although the tick vector is widely distributed (Walker 1974). This paper describes the isolation of *C ruminantium* from eight different areas endemic for *Amblyomma* species in eight Districts of Kenya, revealing the wide geographical range of the organism in the country. The paper also indicates the relative success of, and the problems associated with the four methods used in the isolation attempts.

Materials and methods

Isolation sites

Sixteen sites in 12 different Districts were selected within the known distribution of *Amblyomma* species ticks and thus potential heartwater areas (Fig 1). At a few sites the local veterinary officers reported sporadic suspect heartwater cases or outbreaks of heartwater were known to have occurred in the past.

Materials used in isolation attempts

Blood. – Jugular venous blood samples from healthy sheep and cattle infested with *Amblyomma* species ticks were collected into vacutainer tubes containing dipotassium ethylenediamine tetracetic acid (EDTA) or heparin (Becton Dickinson). The samples were on ice for two to 18 hours, before pooling and inoculation into a susceptible sheep (Table 1). Blood from sheep and cattle was pooled separately.

Nymphal ticks. – Engorged nymphs were collected from cattle from predilection feeding sites for *Amblyomma* nymphs (particularly the lower legs and heels), and allowed to moult into adults and harden at 28°C and 85 per cent relative humidity. After being identified the adults were applied to the backs of susceptible sheep in body patches as described by Heyne and others (1987). When necessary uninfected male colony ticks were added to facilitate feeding. Table 2 gives the details of tick feeds by adults derived from the nymphal collections.

Adult ticks. – Partially fed *Amblyomma* species adults were removed from cattle and identified. Different species were separated and used for the isolation of *C ruminantium* by two methods. In the first method, adults were applied to feed on body patches on susceptible sheep (Heyne and others 1987). The backs of the sheep were shaved and washed with soap and water. After drying, a calico bag was fixed to the wool around the shaved area, and 20 to 60 ticks were secured within each bag in open tubes. After 24 hours the tubes were removed and the attached ticks were allowed to feed to repletion.

In the second method, tick homogenates, (1·5 to 2 ml containing 10 tick equivalents,) were inoculated into a jugular vein. The tick homogenates were prepared only from undamaged ticks; they were washed in several changes of water followed by 70 per cent ethanol and then homogenised by grinding in a small volume of buffered lactose peptone (BLP) in a mortar and pestle. Tick debris was removed by centrifugation at 1000 rpm for five minutes. The

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TABLE 1: Results of attempts to isolate *Cowdria ruminantium* in sheep using pooled blood

Isolation site	Source of blood pool	Volume inoculated (ml)	Time to inoculation (hours)	Confirmation of *C ruminantium*
Galana	25 cows	10	48	No
Galana	10 cull ewes	10	48	No
Suswa	7 cows	10	20	No
Suswa	9 sheep	10	20	No
Tharaka Nithi	4 goats	10	2	No

supernatant was adjusted to four, 10 or 20 tick equivalents/ml with BLP depending on the size of the tick collection. The homogenate was usually used immediately and injected intravenously into a susceptible sheep which had been pre-treated with 1 ml atropine sulphate (Bimeda) subcutaneously 10 minutes earlier. The remaining homogenate was cryopreserved in 10 per cent dimethyl sulphoxide (DMSO; Sigma) and snap frozen in liquid nitrogen.

Tables 2 and 3 show the details of the adult tick feeds and inoculations of homogenates of adult ticks.

Experimental sheep

Corriedale wethers aged between six and eight months from an area non-endemic for *Amblyomma* species were used. They were housed in tick-proof barns and maintained on hay supplemented with concentrate and a mineral block. Water was freely available.

Monitoring infections

Sheep infected by any of the above methods were observed daily for clinical signs of depression, anorexia and nervous signs, and their rectal temperature was recorded. Animals developing clinical signs were euthanased, although some died suddenly before clinical signs developed.

Confirmation of infection

The presence of *C ruminantium* was confirmed by the examination of brain crush smears made post mortem as described by Purchase (1945). The smears were stained with Diff-Quick (Baxter) or Giemsa (Merck) stain and examined for colonies in the cytoplasm of endothelial cells of brain capillaries. When it was not possible to confirm heartwater in the brain crust smears of a reacting sheep, a blood stabilate prepared from the sheep during the pyrexic reaction was inoculated intravenously into a susceptible sheep or goat; this procedure provided confirmation if the animal subsequently developed cowdriosis.

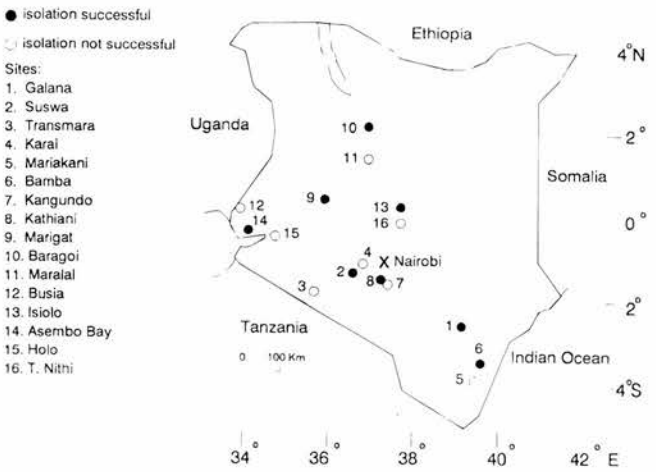

FIG 1: Sites of *Cowdria* isolation attempts in Kenya

Preparation of blood stabilate

When sheep developed fever above 40·5°C, jugular blood was collected into dipotassium EDTA or heparin vacutainer tubes. The blood was chilled to 4°C and DMSO added dropwise to 10 per cent v/v while stirring over crushed ice. The blood-DMSO mixture was immediately divided into subsamples which were snapfrozen in liquid nitrogen.

Results

Isolation sites

Thirteen isolates of *C ruminantium* were made from eight of the 16 sites investigated in eight different Districts of Kenya (Fig 1).

Tick species

Amblyomma variegatum was the sole or predominant *Amblyomma* species found on animals at nine of the sites. *A gemma* was the predominant species at four sites; Isiolo, Baragoi, Mariakani and Bamba. In Maralal *A variegatum* and *A gemma* were found in approximately equal numbers. *A lepidum* was found at two sites: in Galana in approximately equal numbers to *A gemma* and in Isiolo. *A cohaerens* was found only in Transmara. In general, cattle at all the sites which were not subject to tick control had heavy burdens of adult ticks (more than 100 ticks per animal) whereas sheep and goats yielded very few *Amblyomma* species ticks even when they were not under tick

TABLE 2: Results of attempts to isolate *Cowdria ruminantium* in sheep using adult ticks

Isolation site	Tick species	Number of adult ticks applied (collected as engorged nymphs)	Number of ticks applied (reattached adults)	Number of ticks attached	Days to fever (°C) 40	41	Confirmation of *C ruminantium*† (day after infection)
Galana	Al	18F*		6	–	16	+[3](17)
Galana	Ag[1]	27F30M		4	20	–	+(22)
Galana	Ag[2]	18F20M		22	–	–	–
Suswa	Av	6F1M*		2	–	18	+(19)
Karai	Av	3M1F		1	–	–	–
Karai	Av		26	3	–	–	–
Mariakani	Ag		36	NC	–	–	–
Kangundo	Av		60	11	–	–	–
Bamba	Ag		40	NC	13	–	+(21)
Marigat	Av		50	11	–	–	–

*Uninfected male ticks added to facilitate feeding
†Colonies detected in brain capillaries
Av *A variegatum*, Ag *A gemma*, Al *A lepidum*
M Male, F Female, NC Not counted
[1] Nymphs collected from cows, [2] Nymphs collected from calves, [3] Confirmation through subinoculation of blood collected during the early febrile period

TABLE 3: Results of attempts to isolate *Cowdria ruminantium* in sheep using adult tick homogenates

Isolation site	Tick species	Days to fever (°C) 40	41	Confirmation of *C ruminantium*† (day after infection)
Kathiani‡	Av	11	12	+(17)
Kathiani‡	Av	11	12	+(13)
Kathiani‡	Av	13	15	+(18)*
Marigat	Av	16	17	+(18)*
Suswa	Av	12	–	+(14)
Transmara	Av	–	–	No
Isiolo	Al/Ag	12	13	+(13)*
Baragoi‡	Ag	10	13	+(17)
Baragoi‡	Ag	7	9	+(11)
Maralal	Av	–	–	No
Busia	Av	–	–	No
Karai	Av	–	–	No
Asembo bay	Av	–	13	+(13)
Holo	Av	–	–	No

* Sheep euthanased
† Colonies detected in brain capillaries
‡ Although from the same isolation site, different batches of ticks were used
Av *A variegatum*, Ag *A gemma*, Al *A lepidum*

control and when other ticks, for example *Rhipicephalus pulchel lus*, were numerous. Seven of the 13 isolates of *C ruminantium* were derived from *A variegatum*, four from *A gemma*, one from *A lepidum* and one, at Isiolo, was made from a mixture of *A gemma* and *A lepidum* (Table 4).

Isolation methods

No isolates of *C ruminantium* were obtained by the subinoculation of pooled blood from suspected heartwater carriers, and the reattachment of adult ticks collected from the field was successful in only one of five attempts. However, the feeding of adults derived from field nymphs gave three isolates from five attempts, and the inoculation of homogenates derived from field adults gave nine isolates from 14 attempts.

Discussion

The successful isolation of *C ruminantium* from eight different Districts of Kenya demonstrates that this organism is widely distributed across the country and is present in ticks infesting healthy cattle, sheep and goats. In most of the Districts it is likely that a state of endemic stability exists and that sporadic cases or outbreaks occur in association with the introduction of non-indigenous and susceptible animals from non-endemic areas, or when the organism is transmitted to young animals irregularly owing to seasonal tick activity or the use of acaricides (Norval 1988, Uilenberg 1990).

The isolation sites were selected on the basis of the presence of *Amblyomma* species ticks and, in some areas, reports of heartwater cases. At no time during the work were suspect clinical cases of heartwater observed or reported from which *C ruminantium* could have been isolated by the subinoculation of blood, which is considered to be a reliable method of isolation (Du Plessis and Kumm 1971, Jongejan and others 1984). Other workers have obtained clinical cases by grazing susceptible hosts in an endemic area (Mackenzie and van Rooyen 1981) but this requires close monitoring and may be impractical. Subinoculation of blood into mice has been used as a means of transporting *C ruminantium* from a field case to the laboratory (Haig 1952). However, not all isolates are infective for mice (Uilenberg 1983, Mackenzie and McHardy 1987). None of the attempts to isolate *C ruminantium* by subinoculation of blood from cattle or small ruminants infested with *Amblyomma* species, and thus suspected carriers of heartwater (Andrew and Norval 1989), was successful, in spite of the fact that in some cases the ticks collected from the same animals did yield isolates. Except in one case, this blood was inoculated 20 or more hours after it was collected and viability studies have indicated that blood loses significant levels of infectivity by this time (Ilemobade and Blotkamp 1978, Bezuidenhout 1984). Norval and others (1992) observed that the inoculation of blood was successful only when the donor blood possessed high rickettsia levels, such as are considered to occur in clinical cases. It is likely that rickettsaemias are low in the blood of carriers and this, combined with the reduced infectivity, probably accounts for the lack of success of this method of isolation. It was not used in later attempts.

Field ticks are the obvious alternative choice as a source of *C ruminantium*, and the use of adults derived from nymphs engorged on cattle was reasonably successful, yielding an isolate on three of five attempts in spite of the fact that few of the adults attached and fed successfully; the relatively high success rate indicated that there was a high infection rate. This method has the advantage of being the most natural, but it had the disadvantage that it was sometimes difficult to find engorged nymphs during a single visit to a site, probably because they drop off as soon as they are fully engorged and because in some areas some tick control was being practised. Another disadvantage is that it is necessary to wait several weeks for the ticks to moult to adults and then feed before it can be determined whether an isolation attempt has been successful. The Kiswani isolate (Kocan and others 1987) of *C ruminantium* was obtained by this method from adults derived from *A variegatum* nymphs collected at the Kenya Coast (A. D.

TABLE 4: Summary of the attempts to isolate *Cowdria ruminantium*

Site	District	Number of isolates	Derived from Av	Ag	Al	Isolation methods (successful/attempted) blood	adult ex nymphs	adult reattachment	adult homogenate
Galana	Tana River	2		1	1	0/2	2/3	–	–
Suswa	Narok	2	2			0/2	1/1	–	1/1
Transmara	Narok	0				–	–	–	0/1
Karai	Kiambu	0				–	0/1	0/1	0/1
Mariakani	Kilifi	0				–	–	0/1	–
Bamba	Kilifi	1		1		–	–	1/1	–
Kangundo	Machakos	0	0			–	–	0/1	–
Kathiani	Machakos	3	3			–	–	–	3/3
Marigat	Baringo	1	1			–	–	0/1	1/1
Baragoi	Samburu	2		2		–	–	–	2/2
Maralal	Samburu	0				–	–	–	0/1
Busia	Busia	0				–	–	–	0/1
Isiolo	Isiolo	1		Mixed		–	–	–	1/1
Asembo bay	Siaya	1	1			–	–	–	1/1
Holo	Kisumu	0				–	–	–	0/1
Tharaka Nithi	Meru	0				0/1	–	–	–
Total successful attempts		13	7	4 (1 Mixed)	1	0/5	3/5	1/5	9/14

Av *A variegatum*, Ag *A gemma*, Al *A lepidum*
– Not done

Irvin, personal communication). Collecting adult ticks off animals and feeding them on susceptible sheep was difficult because many failed to reattach and those that did caused severe irritation which made the sheep attempt to remove them; only one of five attempts was successful. To avoid these problems, adults collected from the field were inoculated as a ground-up tick homogenate. This approach has been used previously (Theiler and du Toit 1928, Alexander 1931, Bezuidenhout 1982) and was successful on nine out of 14 occasions. Pre-treatment of the recipient sheep with atropine prevented an anaphylactic reaction due to the inoculation of tick homogenate which has been described by Alexander (1931) and Bezuidenhout (1987).

Adult ticks picked off animals in the field may be infective, and the prefeeding of unfed ticks has been reported to increase their infectivity (Bezuidenhout 1987); this effect would have been achieved in a proportion of the adults collected. However, the possible damage to the ticks during their removal, and their poor re-attachment rate, makes the use of tick homogenate more likely to be successful than re-attaching field ticks. Alternatively, adults collected while feeding may have already shed the infective *C ruminantium* that were in their salivary glands, although infective particles may still be in other organs of the same tick (Kocan and Bezuidenhout 1987). The grinding process used during the preparation of tick homogenates may liberate these organisms and improve the chances of isolating the rickettsia.

The use of baits to attract and trap unfed *Amblyomma* species ticks in the field is also likely to help in future isolations of *C ruminantium* (Norval and others 1987).

A variegatum is regarded as the most efficient vector of *C ruminantium* in Kenya (Walker 1974) and seven of the isolates were derived from this species. The four isolates from *A gemma* and the one isolate from *A lepidum* are the first and second reports (Jongejan and others 1984) for these species, respectively, and will be useful in determining whether the infectivity of isolates of *C ruminantium* is highest for the vector species from which it originates. The transtadial transmission of infection in *A gemma* from nymph to adult is also the first report for this species. *A gemma* is widely distributed in the more arid areas of Kenya and overlaps with *A variegatum* in the drier parts of the latter's range (Walker 1974); it is therefore likely to play a significant role in the transmission and epidemiology of heartwater in Kenya.

The distribution of *A variegatum* almost parallels that of *Rhipicephalus appendiculatus*, the vector for *Theileria parva*. With immunisation against East Coast fever (*T parva* infection) soon to be implemented (Kariuki and others 1994), and tick control likely to be reduced, it is possible that *A variegatum*, and thus heartwater infection, may be reintroduced and put cattle and small ruminants in the affected areas at risk of disease. The isolates made during this study are good evidence of the wide distribution of *C ruminantium* in Kenya, and they provide a basis for further studies on the organism and the disease it causes.

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